54

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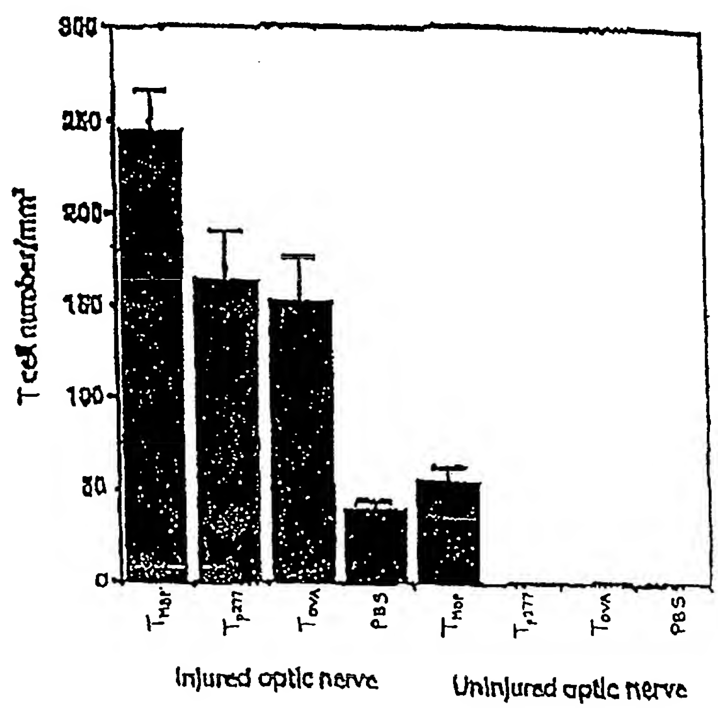
(57) Abstract

Compositions and methods are provided for treating injury to or disease of the central or peripheral nervous system. In one embodiment, treatment is effected using activated T cells that recognize an antigen of the nervous system or a peptide derived therefrom or a derivative thereof to promote nerve regeneration or to prevent or inhibit neuronal degeneration within the nervous system. Treatment involves administering an NS–specific antigen or peptide derived therefrom or a derivative thereof, or a nucleotide sequence encoding said antigen or peptide, to promote nerve regeneration or to prevent or inhibit neuronal degeneration in the nervous system, either the central nervous system or the peripheral nervous system. The NS–specific activated T cells can be administered alone or in combination with NS–specific antigen or peptide derived therefrom or a derivative thereof or a nucleotide sequence encoding said antigen or peptide, or any combination thereof.



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ACTIVATED T CELLS, NERVOUS SYSTEM-SPECIFIC ANTIGENS AND THEIR USES

Field of the Invention

The present invention relates to compositions and methods for the promotion of nerve regeneration or prevention or inhibition of neuronal degeneration to ameliorate the effects of injury or disease of the nervous system (NS). In certain embodiments, activated antiself T cells, an NS-specific antigen or peptide derived therefrom or a nucleotide sequence encoding an NS-specific antigen or peptide derived therefrom can be used to promote nerve regeneration or to prevent or inhibit neuronal degeneration caused by injury or disease of nerves within the central nervous system or peripheral nervous system of a human subject. The compositions of the present invention may be administered alone or may be optionally administered in any desired combination.

Background of the Invention

The nervous system comprises the central (CNS) and the peripheral (PNS) nervous system. The central nervous system is composed of the brain and spinal cord; the peripheral nervous system consists of all of the other neural elements, namely the nerves and ganglia outside of the brain and spinal cord.

Damage to the nervous system may result from a traumatic injury, such as penetrating trauma or blunt trauma, or a disease or disorder, including but not limited to Alzheimer's disease, Parkinson's disease, multiple sclerosis, Huntington's disease, amyotrophic lateral sclerosis (ALS), diabetic neuropathy, senile dementia, and ischemia.

Maintenance of central nervous system integrity is a complex "balancing act" in which compromises are struck with the immune system. In most tissues, the immune system plays an essential part in protection, repair, and healing. In the central nervous system, because of its unique immune privilege, immunological reactions are relatively limited (Streilein, J.W., 1993, Curr. Opin. Immunol. 5:428-423; Streilein, J.W., Science 270:1158-1159). A growing body of evidence indicates

that the failure of the mammalian central nervous system to achieve functional recovery after injury is a reflection of an ineffective dialog between the damaged tissue and the immune system. For example, the restricted communication between the central nervous system and blood-borne macrophages affects the capacity of axotomized axons to regrow; transplants of activated macrophages can promote central nervous system regrowth (Lazarov Spiegler, O., et al, 1996, FASEB J. 19:1296-1302; Rapalino, O. et al., 1998, Nature Med. 4:814-821).

Activated T cells have been shown to enter the central nervous system parenchyma, irrespective of their antigen specificity, but only T cells capable of reacting with a central nervous system antigen seem to persist there (Hickey, W.F. et al., 1991, J. Neurosci. Res. 28:254-260; Werkele, H., 1993, *In* The Blood-Brain Barrier, Pardridge, Ed., Raven Press, Ltd. New York, 67-85; Kramer, R. et al., 1995, Nature Med. 1(11):1162-1166)). T cells reactive to antigens of central nervous system white matter, such as myelin basic protein (MBP), can induce the paralytic disease experimental autoimmune encephalomyelitis (EAE) within several days of their inoculation into naive recipient rats (Ben Nun, A., et al., 1981, Eur. J. Immunol. 11:195-199). Anti-MPB T cells may also be involved in the human disease multiple sclerosis (Ota, K. et al., 1990 Nature 346:183-187; Martin, R. 1997, J. Neural Transm. Suppl. 49:53-67). However, despite their pathogenic potential, anti-MBP T cell clones are present in the immune systems of healthy subjects (Burns, J., et al. 1983, Cell Immunol. 81:435-440; Pette, M. et al., 1990, Proc. Natl. Acad. Sci. USA 87:7968-7972; Martin, R. et al., 1990, J. Immunol. 145:540-548; Schiuesener, H.J, et al., 1985, J. Immunol. 135:3128-3133). Activated T cells, which normally patrol the intact central nervous system, transiently accumulate at sites of central nervous system white matter lesions (Hirschberg, D.L., et al., 1998, J. Neuroimmunol. 89:88-96).

A catastrophic consequence of central nervous system injury is that the primary damage is often compounded by the gradual secondary loss of adjacent neurons that apparently were undamaged, or only marginally damaged, by the initial injury

(Faden, A. I., et al., 1992, Trends Pharmacol. Sci. 13:29-35; Faden, A.I., 1993, Crit. Rev. Neurobiol. 7:175-186; McIntosh, T.K., 1993, J. Neurotrauma 10:215-261). The primary lesion causes changes in extracellular ion concentrations, elevation of amounts of free radicals, release of neurotransmitters, depletion of growth factors, and local inflammation. These changes trigger a cascade of destructive events in the adjacent neurons that initially escaped the primary injury (Lynch, D.R. et al., 1994, Curr. Opin. Neurol. 7:510-516; Bazan, N.G. et al., 1995, J. Neurotrauma 12:791-814; Wu, D. et al., 1994, J.Neurochem. 62:37-44). This secondary damage is mediated by activation of voltage-dependent or agonist-gated channels, ion leaks, activation of calcium-dependent enzymes such as proteases, lipases and nucleases, mitochondrial dysfunction and energy depletion, culminating in neuronal cell death (Yoshina, A. et al., 1991 Brain Res. 561:106-119; Hovda, D.A. et al., 1991, Brain Res. 567:1-10; Zivin, J.A., et al, 1991 Sci. Am. 265:56-63; Yoles, E. et al., 1992, Invest. Ophthalmol. Vis. Sci. 33:3586-3591). The widespread loss of neurons beyond the loss caused directly by the primary injury has been called "secondary degeneration."

Another tragic consequence of central nervous system injury is that neurons in the mammalian central nervous system do not undergo spontaneous regeneration following an injury. Thus, a central nervous system injury causes permanent impairment of motor and sensory functions.

Spinal cord lesions, regardless of the severity of the injury, initially result in a complete functional paralysis known as spinal shock. Some spontaneous recovery from spinal shock may be observed, starting a few days after the injury and tapering off within three to four weeks. The less severe the insult, the better the functional outcome. The extent of recovery is a function of the amount of undamaged tissue minus the loss due to secondary degeneration. Recovery from injury would be improved by neuroprotective treatment that could reduce secondary degeneration.

Citation or identification of any reference in this section or any other part of this application shall not be

construed as an admission that such reference is available as prior art to the invention.

SUMMARY OF THE INVENTION

The present invention is directed to methods and compositions for the promotion of nerve regeneration or prevention or inhibition of neuronal degeneration to ameliorate the effects of injury to or disease of the nervous system (NS). The present invention is based in part on the applicants' unexpected discovery that activated T cells that recognize an antigen of the NS of the patient promote nerve regeneration or confer neuroprotection. As used herein, "neuroprotection" refers to the prevention or inhibition of degenerative effects of injury or disease in the NS. Until recently, it was thought that the immune system excluded immune cells from participating in nervous system repair. It was quite surprising to discover that NS-specific activated T cells can be used to promote nerve regeneration or to protect nervous system tissue from secondary degeneration which may follow damage caused by injury or disease of the CNS or PNS.

"Activated T cell" as used herein includes (i) T cells that have been activated by exposure to a cognate antigen or peptide derived therefrom or derivative thereof and (ii) progeny of such activated T cells. As used herein, a cognate antigen is an antigen that is specifically recognized by the T cell antigen receptor of a T cell that has been previously exposed to the antigen. Alternatively, the T cell which has been previously exposed to the antigen may be activated by a mitogen, such as phytohemagglutinin (PHA) or concanavalin A.

In one embodiment, the present invention provides pharmaceutical compositions comprising a therapeutically effective amount of NS-specific activated T cells and methods for using such compositions to promote nerve regeneration or to prevent or inhibit neuronal degeneration in the CNS or PNS, in an amount which is effective to ameliorate the effects of an injury or disease of the NS. "NS-specific activated T cell" as used herein refers to an activated T cell having specificity for an antigen of the NS of a patient. The antigen used to confer the specificity to the T cells may be a self NS-antigen

of the patient, a peptide derived therefrom, or an NS-antigen of another individual or even another species, or a peptide derived therefrom, as long as the activated T cell recognizes an antigen in the NS of the patient.

The NS-specific activated T cells are used to promote nerve regeneration or to prevent or inhibit the effects of disease. If the disease being treated is an autoimmune disease, in which the autoimmune antigen is an NS antigen, the T cells which are used in accordance with the present invention for the treatment of neural damage or degeneration caused by such disease are preferably not activated against the same autoimmune antigen involved in the disease. While the prior art has described methods of treating autoimmune diseases by administering activated T cells to create a tolerance to the autoimmune antigen, the T cells of the present invention are not administered in such a way as to create tolerance, but are administered in such a way as to create accumulation of the T cells at the site of injury or disease so as to facilitate neural regeneration or to inhibit neural degeneration.

The prior art also discloses uses of immunotherapy against tumors, including brain tumors, by administering T cells specific to an NS antigen in the tumor so that such T cells may induce an immune system attack against the tumors. The present invention is not intended to comprehend such prior art techniques. However, the present invention is intended to comprehend the inhibition of neural degeneration or the enhancement of neural regeneration in patients with brain tumors by means other than the prior art immunotherapy of brain tumors. Thus, for example, NS-specific activated T cells, which are activated to an NS antigen of the patient other than an antigen which is involved in the tumor, would be expected to be useful for the purpose of the present invention and would not have been suggested by known immunotherapy techniques.

The present invention also provides pharmaceutical compositions comprising a therapeutically effective amount of an NS-specific antigen or peptide derived therefrom or derivative thereof and methods of use of such compositions to promote nerve regeneration or to prevent or inhibit neuronal degeneration in the CNS or PNS, in which the amount is

effective to activate T cells *in vivo* or *in vitro*, wherein the activated T cells inhibit or ameliorate the effects of an injury or disease of the NS. "NS-specific antigen" as used herein refers to an antigen that specifically activates T cells such that following activation the activated T cells accumulate at a site of injury or disease in the NS of the patient. In one embodiment, the peptide derived from an NS-specific antigen is a "cryptic epitope" of the antigen. A cryptic epitope activates specific T cells after an animal is immunized with the particular peptide, but not with the whole antigen. In another embodiment, the peptide derived from an NS-specific antigen is an immunogenic epitope of the antigen. "Derivatives" of NS-specific antigens or peptides derived therefrom as used herein refers to analogs or chemical derivatives of such antigens or peptides as described below, see Section 5.2.

The present invention also provides pharmaceutical compositions comprising a therapeutically effective amount of a nucleotide sequence encoding an NS-specific antigen or peptide derived therefrom or derivative thereof and methods of use of such compositions to promote nerve regeneration or for preventing or inhibiting neuronal degeneration in the CNS or PNS in which the amount is effective to ameliorate the effects of an injury or disease of the NS.

In the practice of the invention, therapy for amelioration of effects of injury or disease comprising administration of NS-specific activated T cells may optionally be in combination with an NS-specific antigen or peptide derived therefrom.

Additionally, oral administration of NS-specific antigen or a peptide derived therefrom, can be combined with active immunization to build up a critical T cell response immediately after injury.

In another embodiment cell, banks can be established to store NS sensitized T cells for neuroprotective treatment of individuals at a later time, as needed. In this case, autologous T cells may be obtained from an individual. Alternatively, allogeneic or semi-allogeneic T cells may be stored such that a bank of T cells of each of the most common

MHC-class II types are present. In case an individual is to be treated for an injury, preferably autologous stored T cells are used, but, if autologous T cells are not available, then cells should be used which share an MHC type II molecule with the patient, and these would be expected to be operable in that individual. The cells are preferably stored in an activated state after exposure to an NS antigen or peptide derived therefrom. However, the cells may also be stored in a resting state and activated once they are thawed and prepared for use. The cell lines of the bank are preferably cryopreserved. The cell lines are prepared in any way which is well known in the art. Once the cells are thawed, they are preferably cultured prior to injection in order to eliminate non-viable cells. During this culturing, the cells can be activated or reactivated using the same NS antigen or peptide as used in the original activation. Alternatively, activation may be achieved by culturing in the presence of a mitogen, such as phytohemagglutinin (PHA) or concanavalin A (prefereably the former). This will place the cells into an even higher state of activation. The few days that it takes to culture the cells should not be detrimental to the patient as the treatment in accordance with the present invention may occur any time up to a week or more after the injury in order to still be effective. Alternatively, if time is of the essence, the stored cells may be administered immediately after thawing.

BRIEF DESCRIPTION OF THE FIGURES

Fig. 1 is a bar graph showing the presence of T cells in uninjured optic nerve or in injured optic nerve one week after injury. Adult Lewis rats were injected with activated T cells of the anti-MBP (T_{MBP}), anti-OVA (T_{OVA}), anti-p277 (T_{p277}) lines, or with PBS, immediately after unilateral crush injury of the optic nerve. Seven days later, both the injured and uninjured optic nerves were removed, cryosectioned and analyzed immunohistochemically for the presence of immunolabeled T cells. T cells were counted at the site of injury and at randomly selected areas in the uninjured optic nerves. The histogram shows the mean number of T cells per mm^2 $\pm$ s.e.m., counted in two to three sections of each nerve. Each

group contained three to four rats. The number of T cells was considerably higher in injured nerves of rats injected with anti-MBP, anti-OVA or anti-p277 T cells; statistical analysis (one-way ANOVA) showed significant differences between T cell numbers in injured optic nerves of rats injected with anti-MBP, anti-OVA, or anti-p277 T cells and the T cell numbers in injured optic nerves of rats injected with PBS ($P<0.001$); and between injured optic nerves and uninjured optic nerves of rats injected with anti-MBP, anti-OVA, or anti-p277 T cells ($P<0.001$).

Fig. 2 is a bar graph illustrating that T cells specific to MBP, but not of OVA or p277 or hsp60, protect neurons from secondary degeneration. Immediately after optic nerve injury, rats were injected with anti-MBP, anti-OVA or anti-p277 T cells, or with PBS. The neurotracer dye 4-Di-10-Asp was applied to optic nerves distal to the site of the injury, immediately after injury (for assessment of primary damage) or two weeks later (for assessment of secondary degeneration). Five days after dye application, the retinas were excised and flat-mounted. Labeled retinal ganglion cells (RGCs) from three to five randomly selected fields in each retina (all located at approximately the same distance from the optic disk) were counted by fluorescence microscopy. RGC survival in each group of injured nerves was expressed as the percentage of the total number of neurons spared after the primary injury (42% of neurons remained undamaged after the primary injury). The neuroprotective effect of anti-MBP T cells compared with that of PBS was significant ($P<0.001$, one-way ANOVA). Anti-OVA T cells or anti-p277 T cells did not differ significantly from PBS in their effects on the protection of neurons that had escaped primary injury ($P>0.05$, one-way ANOVA). The results are a summary of five experiments. Each group contained five to ten rats.

Figs. 3 (A-C) present photomicrographs of retrogradely labeled retinas of injured optic nerves of rats. Immediately after unilateral crush injury of their optic nerves, rats were injected with PBS (Fig. 3A) or with activated anti-p277 T cells (Fig. 3B) or activated anti-MBP T cells (Fig. 3C). Two weeks later, the neurotracer dye 4-Di-10-Asp was

applied to the optic nerves, distal to the site of injury. After 5 days, the retinas were excised and flat-mounted. Labeled (surviving) RCGs, located at approximately the same distance from the optic disk in each retina, were photographed.

Figs. 4(A-B) are graphs showing that clinical severity of EAE is not influenced by an optic nerve crush injury. For the results presented in Fig. 4A, Lewis rats, either uninjured (dash line) or immediately after optic nerve crush injury (solid line), were injected with activated anti-MBP T cells. EAE was evaluated according to a neurological paralysis scale. [Data points represent ± s.e.m.] These results represent a summary of three experiments. Each group contained five to nine rats. Fig. 4B shows that the number of RGCs in the uninjured optic nerve is not influenced by injection of anti-MBP T cells. Two weeks after the injection of anti-MBP T cells or PBS, 4-Di-10Asp was applied to the optic nerves. After 5 days the retinas were excised and-flat-mounted. Labeled RGCs from five fields (located at approximately the same distance from the optic disk) in each retina were counted and the average number per mm^2 was calculated. There was no difference between the numbers of labeled RGCs in rats injected with anti-MBP T cells (T_{MBP}) and in PBS-injected control rats.

Fig. 5 is a bar graph showing that T cells specific to p51-70 of MBP protect neurons from secondary degeneration. Immediately after optic nerve injury, rats were injected with anti-MBP T cells, anti-p51-70 T cells, or PBS. The neurotracer dye 4-Di-10-Asp was applied to optic nerves distal to the site of the injury, immediately after injury (for assessment of primary damage) or two weeks later (for assessment of secondary degeneration). Five days after dye application, the retinas were excised and flat-mounted. Labeled retinal ganglion cells (RGCs) from three to five randomly selected fields in each retina (all located at approximately the same distance from the optic disk) were counted by fluorescence microscopy. RGC survival in each group of injured nerves was expressed as the percentage of the total number of neurons spared after primary injury. Compared with

that of PBS treatment, the neuroprotective effects of anti-MBP anti-p51-70 T cells were significant ($P<0.001$, one-way ANOVA).

Figs. 6(A-B) are graphs showing that anti-MBP T cells increase the compound action potential (CAP) amplitudes of injured optic nerves. Immediately after optic nerve injury, rats were injected with either PBS or activated anti-MBP T cells (T_{MBP}). Two weeks later, the CAPs of injured (Fig. 6A) and uninjured (Fig. 6B) nerves were recorded. There were no significant differences in mean CAP amplitudes between uninjured nerves obtained from PBS-injected and T cell-injected rats (n-8; $p=0.8$, Student's t-test). The neuroprotective effect of anti-MBP T cells (relative to PBS) on the injured nerve on day 14 after injury was significant ($n=8$, $p=0.009$, Student's t-test).

Figs. 7(A-B) are graphs showing recovery of voluntary motor activity as a function of time after contusion, with and without injection of autoimmune anti-MBP T cells. (7A) Twelve rats were deeply anesthetized and laminectomized, and then subjected to a contusion insult produced by a 10 gram weight dropped from a height of 50 mm. Six of the rats, selected at random, were then inoculated i.p. with 10^7 anti-MBP T cells and the other six were inoculated with PBS. At the indicated time points, locomotor behavior in an open field was scored by observers blinded to the treatment received by the rats. Results are expressed as the mean values for each group. The vertical bars indicate SEM. Differences tested by repeated ANOVA, including all time points, were significant ($p<0.05$). (7B) A similar experiment using five PBS-treated animals and six animals treated with anti-MBP T cells were all subjected to a more severe contusion. At the indicated time points, locomotor behavior in an open field was scored. The results are expressed as the mean values for each group. The vertical bars indicate S.E.M. Rats in the treated group are represented by open circles and rats in the control group are represented by black circles. Horizontal bars show the median values. The inset shows the median plateau values of the two groups.

Figs 8(A-C) show retrograde labeling of cell bodies at the red nucleus in rats treated with autoimmune anti-MBP T cells (8A) and in control injured (8B) rats. Three months

after contusion and treatment with anti-MBP T cells, some rats from both the treated and the control groups were re-anesthetized and a dye was applied below the site of the contusion. After five to seven days the rats were again deeply anesthetized and their brains were excised, processed, and cryosectioned. Sections taken through the red nucleus were inspected and analyzed qualitatively and quantitatively under fluorescent and confocal microscopes. Significantly, more labelled nuclei were seen in the red nuclei of rats treated with anti-MBP T cells (8A) than in the red nuclei of PBS-treated rats (8B). The quantitative differences are shown in the bar graph (8C) and were obtained from animals with scores of 10 and 11 in the T cell treated group and scores of 6 in the control group. The bar graph shows mean $\pm$ SD.

Fig. 9 is a series of photographs showing diffusion-weighted imaging of contused spinal cord treated with anti-MBP T cells. Spinal cords of MBP-T cell-treated and PBS-treated animals (with locomotion scores of 10 and 8, respectively) were excised under deep anesthesia, immediately fixed in 4% paraformaldehyde solution, and placed into 5 mm NMR tubes. Diffusion anisotropy was measured in a Bruker DMX 400 widebore spectrometer using a microscopy probe with a 5-mm Helmholtz coil and actively shielded magnetic field gradients. A multislice pulsed gradient spin echo experiment was performed with 9 axial slices, with the central slice positioned at the center of the spinal injury. Images were acquired with TE of 31 ms, TR of 2000 ms, a diffusion time of 15 ms, a diffusion gradient duration of 3 ms, field of view 0.6 mm, matrix size 128 x 128, slice thickness 0.5 mm, and slice separation of 1.18 mm. Four diffusion gradient values of 0, 28, 49, and 71 g/cm were applied along the read direction (transverse diffusion) or along the slice direction (longitudinal diffusion). Diffusion anisotropy is manifested by increased signal intensity in the images with the highest transverse diffusion gradient relative to the longitudinal diffusion gradient. The excised spinal cords of a PBS-treated rat and in the rat treated with MBP-T cells were subjected to diffusion-weighted MRI analysis. In the PBS-treated injured control, diffusion anisotropy was seen mainly in sections near the proximal and distal stumps of the

cord, with low anisotropy in sections taken through the site of injury. In contrast, in the treated rat, higher levels of diffusion anisotropy can be seen in sections taken through the site of injury.

Fig. 10 is a graph illustrating inhibition of secondary degeneration after optic nerve crush injury in adult rats. See text, Section 8, for experimental details. Rats were injected intradermally through the footpads with a 21-mer peptide based on amino acid residues 35-55 (MOG p35-55) of myelin/oligodendrocyte glycoprotein (chemically synthesized at the Weizmann Institute, Israel) (50μ/animal) or PBS ten days prior to optic nerve crush injury or MOG p35-55 in the absence of crush injury. MOG p35-55 was administered with Incomplete Freund's Adjuvant. Surviving optic nerve fibers were monitored by retrograde labeling of retinal ganglion cells (RGCs). The number of RGCs in rats injected with PBS or MOG p35-55 was expressed as a percentage of the total number of neurons in rats injected with MOG p35-55 in the absence of crush injury.

Fig. 11 is a graph illustrating inhibition in adult rats of secondary degeneration after optic nerve crush injury by MBP. See text, Section 9, for experimental details. MBP (Sigma, Israel) (1 mg in 0.5 ml saline) was administered orally to adult rats by gavage using a blunt needle. MBP was administered 5 times, i.e., every third day beginning two weeks prior to optic nerve crush injury. Surviving optic nerve fibers were monitored by retrograde labeling of retinal ganglion cells (RGCs). The number of RGCs in treated rats was expressed as a percentage of the total number of neurons in untreated rats following the injury.

Figs. 12 (A-F) show expression of B7 costimulatory molecules in intact and injured rat optic nerve. Optic nerves were excised from adult Lewis rats before (12A, 12B) and three days after injury (12C, 12D, 12E) and analyzed immunohistochemically for expression of the B7 costimulatory molecule. The site of injury was delineated by GFAP staining. Using calibrated cross-action forceps, the right optic nerve was subjected to a mild crush injury 1-2 mm from the eye. The uninjured cointralateral nerve was left undisturbed. Immunohistochemical analysis of optic nerve antigens was

performed as follows. Briefly, longitudinal cryosections of the excised nerves (20 μm thick) were picked up onto gelatin-coated glass and fixed with ethanol for ten minutes at room temperature. The sections were washed and incubated for one hour at room temperature with mouse monoclonal antibody to rat GFAP (BioMakor, Israel), diluted 1:100, and with antibodies to B7.2 costimulatory molecule and the B7.1 costimulatory molecule (PHARMINGEN, San Diego, CA), diluted 1:25. The sections were washed again and incubated with rhodamine isothiocyanate-conjugated goat anti-mouse IgG (with minimal cross-reaction to rat, human, bovine and horse serum protein) (Jackson ImmunoResearch, West Grove, PA), for one hour at room temperature. All washing solutions contained PBS and 0.05% Tween-20. All diluting solutions contained PBS containing 3% fetal calf serum and 2% bovine serum albumin. The sections were treated with glycerol containing 1,4-diazobicyclo-(2,2,2)-octane and were then viewed with a Zeiss microscope. Note the morphological changes of the B7.2 positive cells after injury, from a rounded (12A, 12B) to a star-like shape (12C, 12D). The B7.2 positive cells were present at a higher density closer to the injury site (12E). Expression of B7.1 was detectable only from day seven and only at the injured site (12F).

Figs. 13 A-C show immunohistochemical analysis of T cells, macrophages or microglia, and B7.2 costimulatory molecules in the injured optic nerves of rats fed MBP. Lewis rats aged 6-8 weeks were fed 1 mg of bovine MBP (Sigma, Israel) (2 mg MBP/ml PBS) or 0.5 ml PBS only every other day by gastric intubation using a stainless steel feeding needle (Thomas Scientific, Swedesboro, NJ) (Chen, Y., Kuchroo, V.K.,. Inobe, J. Hafler, D.A. & Weiner, H.L. Regulatory T cell clones induced by oral tolerance: suppression of autoimmune encephalomyelitis. <u>Science</u> 265:1237-1240, 1994). Ten days after starting MBP the right optic nerves were subjected to calibrated crush injury, as described for Figure 12. Three days later the nerves were excised and prepared for immunohistochemical analysis of T cells using mouse monoclonal antibodies to T cell receptor 11, diluted 1:25, macrophages or microglia using anti-ED1 antibodies (Serotek, Oxford, U.K) diluted 1:250, astrocytes using anti-GFAP antibodies and B7.2

costimulatory molecules as described for Fig. 12. There were no significant quantitative differences in T cells or in ED-1 positive cells between injured optic nerves of PBS-fed (13A) and MBP-fed (13B) rats. The number of B7.2 positive cells at the site of injury of MBP-fed rats (13C) should be noted, as compared with injured controls (Fig. 12E).

Fig. 14 is a graph showing the slowing of neuronal degeneration in rats with orally induced tolerance to MBP. Lewis rats were fed 1 mg MBP daily, or every other day, or 4 times a day at two hour intervals for five consecutive days. Control animals were given PBS or the non-self antigen OVA (Sigma, Israel). Ten days after the start of MBP ingestion, the right optic nerves were subjected to a calibrated mild crush injury. Two weeks later the RGCs were retrogradely labelled by application of the fluorescent lipophilic dye, 4-(4-didecylamino)styryl)-N-methylpyridinium iodide (4-Di-10-Asp) (Molecular Probes Europe BV, Netherlands), distally to the site of injury, as described. Briefly, complete axotomy was performed 1-2 mm from the distal border to the injury site, and solid crystals (0.2-0.4 mm in diameter) of 4-Di-10-Asp were immediately deposited at the site of the lesion. Retrograde labelling of RGCs by the dye gives a reliable indication of the number of still-functioning neurons, as only intact axons can transport the dye to their cell bodies in the retina. Six days after dye application, the retina was detached from the eye, prepared as a flattened whole mount in 4% paraformaldehyde solution, and examined for labelled ganglion cells by fluorescence microscopy. RGCs were counted from three different regions in the retina. The results are expressed as normalized percentage of each retina to untreated injured animal mean of the same experiment. The median of each group is shown as a bar (Control vs. MBP OTx4 ** P<0.01; Control vs. MBP OT ** P, 0.01; Control vs. OVA OT ns P>0.05.

Fig. 15 shows the nucleotide sequence of rat myelin basic protein gene, SEQ ID NO:1, Genbank accession number M25889 (Schaich et al., Biol. Chem. 367:825-834, 1986).

Fig. 16 shows the nucleotide sequence of human myelin basic protein gene, SEQ ID NO:2, Genbank accession number

M13577 (Kamholz et al., Proc. Natl. Acad. Sci. U.S.A. 83(13): 4962-4966, 1986).

Figs 17 (A-F) show the nucleotide sequences of human myelin proteolipid protein gene exons 1-7, SEQ ID NOs:3-8, respectively, Genbank accession number M15026-M15032 respectively (Diehl et al., Proc. Natl. Acad Sci. U.S.A. 83(24):9807-9811, 1986; published erratum appears in Proc Natl Acad Sci U.S.A. 86(6):617-8, 1991).

Fig. 18 shows the nucleotide sequence of human myelin oligodendrocyte glycoprotein gene, SEQ ID NO:9, Genbank accession number Z48051 (Roth et al., submitted (17-Jan-1995) Roth, CNRS UPR 8291, CIGH, CHU Purpan, Toulouse, France, 31300; Gonzalez et al., Mol. Phylogent. Evol. 6:63-71, 1996).

Fig. 19 shows the nucleotide sequence of rat proteolipid protein and variant, SEQ ID NO:10, Genbank accession number M16471 (Nave et al, Proc. Natl. Acad. Sci. U.S.A 84:600-604, 1987).

Fig. 20 shows the nucleotide sequence of rat myelin-associated glycoprotein, SEQ ID NO:11, Genbank accession number M14871 (Arquint et al, Proc. Natl. Acad. Sci. USA 84:600-604, 1987).

Fig. 21 shows the amino acid sequence of human myelin basic protein, SEQ ID NO:12, Genbank accession number 307160 (Kamholz et al., 1986, Proc. Natl. Acad. Sci. U.S.A. 83(13):4962-4966, 1986).

Fig. 22 shows the amino acid sequence of human proteolipid protein, SEQ ID NO:13, Genbank accession number 387028.

Fig. 23 shows the amino acid sequence of human myelin oligodendrocyte glycoprotein, SEQ ID NO:14, Genbank accession number 793839 (Roth et al., Genomics 28(2):241-250, 1995; Roth Submitted (17-JAN-1995) Roth CNRS UPR 8291, CIGH, CHU Purpan, Toulouse, France, 31300; Gonzalez et al., Mol. Phylogent. Evol. 6:63-71, 1996).

DETAILED DESCRIPTION OF THE INVENTION

Merely for ease of explanation, the detailed description of the present invention is divided into the following subsections: (1) NS-specific activated T cells; (2)

NS-specific antigens, peptides derived therefrom and derivatives thereof; (3) nucleotide sequences encoding NS-specific antigens and peptides derived therefrom; (4) therapeutic uses of non-recombinant, NS-specific activated T cells, NS-specific antigens, peptides derived therefrom and derivatives thereof, and nucleotide sequences encoding NS-specific antigens and peptides derived therefrom; and (5) formulations and modes of administration of nonrecombinant, NS-specific activated T cells, NS-specific antigens, peptides derived therefrom and derivatives thereof, and nucleotide sequences encoding NS-specific antigens and peptides derived therefrom.

5.1 NS-SPECIFIC ACTIVATED T CELLS

NS-specific activated T cells (ATCs) can be used for ameliorating or inhibiting the effects of injury or disease of the CNS or PNS that result in NS degeneration or for promoting regeneration in the NS, in particular the CNS.

The NS-specific activated T cells are preferably autologous, most preferably of the CD4 and/or CD8 phenotypes, but they may also be allogeneic T cells from related donors, e.g., siblings, parents, children, or HLA-matched or partially matched, semi-allogeneic or fully allogeneic donors.

In addition to the use of autologous T cells isolated from the subject, the present invention also comprehends the use of semi-allogeneic T cells for neuroprotection. These T cells may be prepared as short- or long-term lines and stored by conventional cryopreservation methods for thawing and administration, either immediately or after culturing for 1-3 days, to a subject suffering from injury to the central nervous system and in need of T cell neuroprotection.

The use of semi-allogeneic T cells is based on the fact that T cells can recognize a specific antigen epitope presented by foreign antigen presenting cells (APC), provided that the APC express the MHC molecule, class I or class II, to which the specific responding T cell population is restricted, along with the antigen epitope recognized by the T cells. Thus, a semi-allogeneic population of T cells that can recognize at least one allelic product of the subject's MHC

molecules, preferably an HLA-DR or an HLA-DQ or other HLA molecule, and that is specific for a NS-associated antigen epitope, will be able to recognize the NS antigen in the subject's area of NS damage and produce the needed neuroprotective effect. There is little or no polymorphism in the adhesion molecules, leukocyte migration molecules, and accessory molecules needed for the T cells to migrate to the area of damage, accumulate there, and undergo activation. Thus, the semi-allogeneic T cells will be able to migrate and accumulate at the CNS site in need of neuroprotection and will be activated to produce the desired effect.

It is known that semi-allogeneic T cells will be rejected by the subject's immune system, but that rejection requires about two weeks to develop. Hence, the semi-allogeneic T cells will have the two week window of opportunity needed to exert neuroprotection. After two weeks, the semi-allogeneic T cells will be rejected from the body of the subject, but that rejection is advantageous to the subject because it will rid the subject of the foreign T cells and prevent any untoward consequences of the activated T cells. The semi-allogeneic T cells thus provide an important safety factor and are a preferred embodiment.

It is known that a relatively small number of HLA class II molecules are shared by most individuals in a population. For example, about 50% of the Jewish population express the HLA-DR5 gene. Thus, a bank of specific T cells reactive to NS antigen epitopes that are restricted to HLA-DR5 would be useful in 50% of that population. The entire population can be covered essentially by a small number of additional T cell lines restricted to a few other prevalent HLA molecules, such as DR1, DR4, DR2, etc. Thus, a functional bank of uniform T cell lines can be prepared and stored for immediate use in almost any individual in a given population. Such a bank of T cells would overcome any technical problems in obtaining a sufficient number of specific T cells from the subject in need of neuroprotection during the open window of treatment opportunity. The semi-allogeneic T cells will be safely rejected after accomplishing their role of neuroprotection. This aspect of the invention does not

contradict, and is in addition to the use of autologous T cells as described herein.

The NS-specific activated T cells are preferably non-attenuated, although attenuated NS-specific activated T cells may be used. T cells may be attenuated using methods well known in the art, including but not limited to, by gamma-irrdiation, e.g., 1.5-10.0 Rads (Ben-Nun, A., Wekerle, H. and Cohen, I.R., Nature 292:60-61 (1981); Ben-Nun, A. and Cohen, I.R., J. Immunol. 129:303-308 (1982)); and/or by pressure treatment, for example as described in U.S. Patent No. 4,996,194 (Cohen et al.); and/or by chemical cross-linking with an agent such as formaldehyde, glutaraldehyde and the like, for example as described in U.S Patent No. 4,996,194 (Cohen et al.); and/or by cross-linking and photoactivation with light with a photoactivatable psoralen compound, for example as described in U.S. Patent No. 5,114,721 (Cohen et al.); and/or by a cytoskeletal disrupting agent such as cytochalsin and colchicine, for example as described in U.S. Patent No. 4,996,194 (Cohen et al.). In a preferred embodiment the NS-specific activated T cells are isolated as described below. T cells can be isolated and purified according to methods known in the art (Mor and Cohen, 1995, J. Immunol. 155:3693-3699). For an illustrative example, see Section 6.1.

Circulating T cells of a subject which recognize myelin basic protein or another NS antigen, such as the amyloid precursor protein, are isolated and expanded using known procedures. In order to obtain NS-specific activated T cells, T cells are isolated and the NS-specific ATCs are then expanded by a known procedure (Burns et al., Cell Immunol. 81:435, 1983; Pette et al., Proc. Natl. Acad. Sci. USA 87:7968, 1990; Mortin et al., J. Immunol. 145:540, 1990; Schluesener et al., J. Immunol. 135:3128, 1985; Suruhan-Dires Keneli et al., Euro. J. Immunol. 23:530, 1993, which are incorporated herein by reference in their entirety).

The isolated T cells may be activated by exposure of the cells to one or more of a variety of natural or synthetic NS-specific antigens or epitopes, including but not limited to, myelin basic protein (MBP), myelin oligodendrocyte glycoprotein (MOG), proteolipid protein (PLP), myelin-associated

glycoprotein (MAG), S-100, β-amyloid, Thy-1, P0, P2 and neurotransmitter receptors. In a preferred embodiment, the isolated T cells are activated by one or more cryptic epitopes, including but limited to the following MBP peptides: p11-30, p51-70, p91-110, p131-150, and p-151-170.

During *ex vivo* activation of the T cells, the T cells may be activated by culturing them in medium to which at least one suitable growth promoting factor has been added. Growth promoting factors suitable for this purpose include, without limitation, cytokines, for instance tumor necrosis factor α (TNF-α), interleukin 2 (IL-2), and interleukin 4 (IL-4).

In one embodiment, the activated T cells endogenously produce a substance that ameliorates the effects of injury or disease in the NS.

In another embodiment, the activated T cells endogenously produce a substance that stimulates other cells, including, but not limited to, transforming growth factor-β (TGF-β), nerve growth factor (NGF), neurotrophic factor 3(NT-3), neurotrophic factor 4/5 (NT-4/5), brain derived neurotrophic factor (BDNF); interferon-γ (IFN-γ), and interleukin-6 (IL-6), wherein the other cells, directly or indirectly, ameliorate the effects of injury or disease.

Following their proliferation *in vitro*, the T cells are administered to a mammalian subject. In a preferred embodiment, the T cells are administered to a human subject. T cell expansion is preferably performed using peptides corresponding to sequences in a non-pathogenic, NS-specific, self protein.

A subject can initially be immunized with an NS-specific antigen using a non-pathogenic peptide of the self protein. A T cell preparation can be prepared from the blood of such immunized subjects, preferably from T cells selected for their specificity towards the NS-specific antigen. The selected T cells can then be stimulated to produce a T cell line specific to the self-antigen (Ben-Nun et al., J. Immunol. 129:303, 1982).

The NS-specific antigen may be a purified antigen or a crude NS preparation, as will be described below. NS-

specific antigen activated T cells, obtained as described above, can be used immediately or may be preserved for later use, e.g., by cryopreservation as described below. NS-specific activated T cells may also be obtained using previously cryopreserved T cells, i.e., after thawing the cells, the T cells may be incubated with NS-specific antigen, optimally together with thymocytes, to obtain a preparation of NS-specific ATCs.

As will be evident to those skilled in the art, the T cells can be preserved, e.g., by cryopreservation, either before or after culture.

Cryopreservation agents which can be used include but are not limited to dimethyl sulfoxide (DMSO) (Lovelock and Bishop, Nature 183:1394-1395, 1959; Ashwood-Smith, Nature 190:1204-1205, 1961), glycerol, polyvinylpyrrolidone (Rinfret, Ann. N.Y. Acad. Sci. 85:576, 1960), polyethylene glycol (Sloviter and Ravdin, Nature 196:548, 1962), albumin, dextran, sucrose, ethylene glycol, i-erythritol, D-ribitol, D-mannitol (Rowe et al., Fed. Proc. 21:157, 1962), D-sorbitol, i-inositol, D-lactose, choline chloride (Bender et al., J. Appl. Physiol. 15:520, 1960), amino acids (Phan The Tran and Bender, Exp. Cell Res. 20:651, 1960), methanol, acetamide, glycerol monoacetate (Lovelock, Biochem. J. 56:265, 1954), inorganic salts (Phan The Tran and Bender, Proc. Soc. Exp. Biol. Med. 104:388, 1960; Phan The Tran and Bender, 1961, in Radiobiology, Proceedings of the Third Australian Conference on Radiobiology, Ilbery, P.L.T., ed., Butterworth, London, p. 59), and DMSO combined with hydroxyethyl starch and human serum albumin (Zaroulis and Leiderman, Cryobiology 17:311-317, 1980).

A controlled cooling rate is critical. Different cryoprotective agents (Rapatz et al., Cryobiology 5(1):18-25, 1968) and different cell types have different optimal cooling rates. See, e.g., Rowe and Rinfret, Blood 20:636 (1962); Rowe, Cryobiology 3(1):12-18 (1966); Lewis et al., Transfusion 7(1):17-32 (1967); and Mazur, Science 168:939-949 (1970) for effects of cooling velocity on survival of cells and on their transplantation potential. The heat of fusion phase where water turns to ice should be minimal. The cooling procedure

can be carried out by use of, e.g., a programmable freezing device or a methanol bath procedure.

Programmable freezing apparatuses allow determination of optimal cooling rates and facilitate standard reproducible cooling. Programmable controlled-rate freezers such as Cryomed or Planar permit tuning of the freezing regimen to the desired cooling rate curve.

After thorough freezing, cells can be rapidly transferred to a long-term cryogenic storage vessel. In one embodiment, samples can be cryogenically stored in mechanical freezers, such as freezers that maintain a temperature of about -80°C or about -20°C. In a preferred embodiment, samples can be cryogenically stored in liquid nitrogen (-196°C) or its vapor. Such storage is greatly facilitated by the availability of highly efficient liquid nitrogen refrigerators, which resemble large Thermos containers with an extremely low vacuum and internal super insulation, such that heat leakage and nitrogen losses are kept to an absolute minimum.

Considerations and procedures for the manipulation, cryopreservation, and long term storage of T cells can be found, for example, in the following references, incorporated by reference herein: Gorin, Clinics in Haematology 15(1):19-48 (1986); Bone-Marrow Conservation, Culture and Transplantation, Proceedings of a Panel, Moscow, July 22-26, 1968, International Atomic Energy Agency, Vienna, pp. 107-186.

Other methods of cryopreservation of viable cells, or modifications thereof, are available and envisioned for use, e.g., cold metal-mirror techniques. See Livesey and Linner, Nature 327:255 (1987); Linner et al., J. Histochem. Cytochem. 34(9):1123-1135 (1986); see also U.S. Patent No. 4,199,022 by Senken et al., U.S. Patent No. 3,753,357 by Schwartz, U.S. Patent No. 4,559,298 by Fahy.

Frozen cells are preferably thawed quickly (e.g., in a water bath maintained at 37-47°C) and chilled immediately upon thawing. It may be desirable to treat the cells in order to prevent cellular clumping upon thawing. To prevent clumping, various procedures can be used, including but not limited to the addition before or after freezing of DNAse (Spitzer et al., Cancer 45:3075-3085, 1980), low molecular

weight dextran and citrate, citrate, hydroxyethyl starch (Stiff et al., Cryobiology 20:17-24, 1983), or acid citrate dextrose (Zaroulis and Leiderman, Cryobiology 17:311-317, 1980), etc.

The cryoprotective agent, if toxic in humans, should be removed prior to therapeutic use of the thawed T cells. One way in which to remove the cryoprotective agent is by dilution to an insignificant concentration.

Once frozen T cells have been thawed and recovered, they are used to promote neuronal regeneration as described herein with respect to non-frozen T cells. Once thawed, the T cells may be used immediately, assuming that they were activated prior to freezing. Preferably, however, the thawed cells are cultured before injection to the patient in order to eliminate non-viable cells. Furthermore, in the course of this culturing over a period of about one to three days, an appropriate activating agent can be added so as to activate the cells, if the frozen cells were resting T cells, or to help the cells achieve a higher rate of activation if they were activated prior to freezing. Usually, time is available to allow such a culturing step prior to administration as the T cells may be administered as long as a week after injury, and possibly longer, and still maintain their neuroregenerative and neuroprotective effect.

5.2 NS-SPECIFIC ANTIGENS AND PEPTIDES DERIVED THEREFROM

Pharmaceutical compositions comprising an NS-specific antigen or peptide derived therefrom or derivative thereof can be used for preventing or inhibiting the effects of injury or disease that result in NS degeneration or for promoting nerve regeneration in the NS, particularly in the CNS. Additionally, NS-specific antigens or peptides derived therefrom or derivatives thereof may be used for *in vivo* or *in vitro* activation of T cells. In one embodiment, the NS-specific antigen is an isolated or purified antigen. In another embodiment, methods of promoting nerve regeneration or of preventing or inhibiting the effects of CNS or PNS injury or disease comprise administering NS-specific antigen or a peptide derived therefrom or derivative thereof to a mammal wherein the

NS-specific antigen or peptide derived therefrom or derivative thereof activates T cells *in vivo* to produce a population of T cells that accumulate at a site of injury or disease of the CNS or PNS.

The NS-specific antigen may be an antigen obtained from NS tissue, preferably from tissue at a site of CNS injury or disease. The NS-specific antigen may be isolated and purified by standard methods including chromatography (e.g., ion exchange, affinity, and sizing column chromatography), centrifugation, differential solubility, or by any other standard technique for the purification of antigens. The functional properties may be evaluated using any suitable assay. In the practice of the invention, natural or synthetic NS-specific antigens or epitopes include, but are not limited to, MBP, MOG, PLP, MAG, S-100, β-amyloid, Thy-1, P0, P2 and a neurotransmitter receptor.

Specific illustrative examples of useful NS-specific antigens include but are not limited to, human MBP, depicted in Fig. 21, (SEQ ID NO:12); human proteolipid protein, depicted in Fig. 22 (SEQ ID NO:13); and human oligodendrocyte glycoprotein, depicted in Fig. 23 (SEQ ID NO:14).

In a preferred embodiment, peptides derived from NS-specific, self-antigens or derivatives of NS-specific antigens activate T cells, but do not induce an autoimmune disease. An example of such peptide is a peptide comprising amino acids 51-70 of myelin basic protein (residues 51-70 of SEQ ID NO:12).

In addition, an NS-specific antigen may be a crude NS-tissue preparation, e.g., derived from NS tissue obtained from mammalian NS. Such a preparation may include cells, both living or dead cells, membrane fractions of such cells or tissue, etc.

an NS-specific antigen may be obtained by an NS biopsy or necropsy from a mammal including, but not limited to, from a site of CNS injury; from cadavers; from cell lines grown in culture. Additionally, an NS-specific antigen may be a protein obtained by genetic engineering, chemically synthesized, etc.

In addition to NS-specific antigens, the invention also relates to peptides derived from NS-specific antigens or

derivatives including chemical derivatives and analogs of NS-specific antigens which are functionally active, i.e., they are capable of displaying one or more know functional activities associated with a full-length NS-specific antigen. Such functional activities include but are not limited to antigenicity (ability to bind (or compete with an NS-antigen for binding) to an anti-NS-specific antibody), immunogenicity (ability to generate antibody which binds to an NS-specific protein), and ability to interact with T cells, resulting in activation comparable to that obtained using the corresponding full-length antigen. The crucial test is that the antigen which is used for activating the T cells causes the T cells to be capable of recognizing an antigen in the NS of the mammal (patient) being treated.

A peptide derived from a CNS-specific or PNS-specific antigen preferably has a sequence comprised within the antigen sequence and is either: (1) an immunogenic peptide, i.e., a peptide that can elicit a human T cell response detected by a T cell proliferation or by cytokine (e.g. interferon (IFN)-γ, interleukin (IL)-2, IL-4 or IL-10) production or (2) a "cryptic epitope" (also designated herein as "immunosilent" or "nonimmunodominant" epitope), i.e., a peptide that by itself can induce a T cell immune response that is not induced by the whole antigen protein (see Moalem et al., Nature Med. 5(1), 1999). Cryptic epitopes for use in the present invention include, but are not limited to, peptides of the myelin basic protein sequence: peptide p11-30, p51-70, p91-110, p131-150, and p151-170. Other peptides can be identified by their capacity to elicit a human T cell response detected by T cell proliferation or by cytokine (e.g. IFN-γ, IL-2, IL-4, or IL-10) production. Such cryptic epitopes are particularly preferred as T cells activated thereby will accumulate at the injury site, in accordance with the present invention, but are particularly weak in autoimmunity. Thus, they would be expected to have fewer side effects.

In one specific embodiment of the invention, peptides consisting of or comprising a fragment of an NS-specific antigen consisting of at least 10 (contiguous) amino acids of the NS-specific antigen are provided. In other embodiments, the

fragment consists of at least 20 contiguous amino acids or 50 contiguous amino acids of the NS-specific antigen. Derivatives of an NS-specific antigen also include but are not limited to those molecules comprising regions that are substantially homologous to the full-length antigen or fragments thereof (e.g., in various embodiments, at least 60% or 70% or 80% or 90% or 95% identity over an amino acid sequence of identical size or when compared to an aligned sequence in which the alignment is done by a computer homology program known in the art) or whose encoding nucleic acid is capable of hybridizing to a coding nucleotide sequence of the full-length NS-specific antigen, under high stringency, moderate stringency, or low stringency conditions.

Computer programs for determining homology may include but are not limited to TBLASTN, BLASTP, FASTA, TFASTA, and CLUSTALW (Pearson and Lipman, Proc. Natl. Acad. Sci. USA 85(8):2444-8, 1988; Altschul et al., J. Mol. Biol. 215(3):40310, 1990; Thompson, et al., Nucleic Acids Res. 22(22):4673-80, 1994; Higgins, et al., Methods Enzymol 266:383-402, 1996; Altschul, et al., 1990, J. Mol. Biol. 215(3):403-410, 1990).

The NS-specific antigen derivatives of the invention can be produced by various methods known in the art. The manipulations which result in their production can occur at the gene or protein level. For example, a cloned gene sequence can be modified by any of numerous strategies known in the art (Maniatis, T., 1990, Molecular Cloning, A Laboratory Manual, 2d ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, New York). The sequence can be cleaved at appropriate sites with restriction endonuclease(s), followed by further enzymatic modification if desired, isolated, and ligated *in vitro*.

Additionally, the coding nucleic acid sequence can be mutated *in vitro* or *in vivo*, to create and/or destroy translation, initiation, and/or termination sequences, or to create variations in coding regions and/or form new restriction endonuclease sites or destroy preexisting ones, to facilitate further *in vitro* modification. Any technique for mutagenesis known in the art can be used, including but not limited to,

chemical mutagenesis, *in vitro* site-directed mutagenesis (Hutchinson, C., et al., J. Biol. Chem 253:6551, 1978), etc.

Manipulations may also be made at the protein level. Included within the scope of the invention are derivatives which are differentially modified during or after translation, e.g., by glycosylation, acetylation, phosphorylation, amidation, derivatization by known protecting/blocking groups, proteolytic cleavage, linkage to an antibody molecule or other cellular ligand, etc. Any of numerous chemical modifications may be carried out by known techniques, including but not limited to specific chemical cleavage by cyanogen bromide, trypsin, chymotrypsin, papain, V8 protease, $NaBH_4$; acetylation, formylation, oxidation, reduction; metabolic synthesis in the presence of tunicamycin; etc.

In addition, derivatives of an NS-specific antigen can be chemically synthesized. For example, a peptide corresponding to a portion of an antigen which comprises the desired domain or which mediates the desired activity can be synthesized by use of a peptide synthesizer. Furthermore, if desired, nonclassical amino acids or chemical amino acids analogs can be introduced as a substitution or addition into the amino acid sequence. Non-classical amino acids include but are not limited to the D-isomers of the common amino acids, α-amino isobutyric acid; 4-aminobutyric acid, Abu; 2-amino butyric acid, γ-Abu; ε-Ahx, 6-amino hexanoic acid; Aib, 2-amino isobutyric acid; 3-amino propionic acid; ornithine; norleucine; novaline; hydroxyproline; sarcosine; citrulline; cysteic acid; t-butylglycine; t-butylalanine; phenylgylcine; cyclohexylalanine; β-alanine; fluoro-amino acids; designer amino acids such as β-methyl amino acids, Cα-methyl amino acids, Nα-methyl amino acids, and amino acid analogs in general. Furthermore, the amino acid can be D (dextrorotary) or L (levorotary).

The functional activity of NS-specific antigens and peptides derived therefrom and derivatives thereof can be assayed by various methods known in the art, including, but not limited to, T cell proliferation assays (Mor and Cohen, J. Immunol. 155:3693-3699, 1995).

An NS-specific antigen or peptide derived therefrom or derivative thereof may be kept in solution or may be provided in a dry form, e.g. as a powder or lyophilizate, to be mixed with appropriate solution prior to use.

5.3 NUCLEOTIDE SEQUENCES ENCODING NS-ANTIGENS AND PEPTIDES DERIVED THEREFROM

Compositions comprising a nucleotide sequence encoding an NS-specific antigen or peptide derived therefrom can be used for preventing or inhibiting the effects of injury or disease that result in CNS or PNS degeneration or for promoting nerve regeneration in the CNS or PNS. Specific illustrative examples of useful nucleotide sequences encoding NS-specific antigens or peptides derived from an NS-specific antigen, include but are not limited to nucleotide sequences encoding rat myelin basic protein (MBP) peptides, depicted in Fig. 15 (SEQ ID NO:1); human MBP, depicted in Fig. 16 (SEQ ID NO:2); human myelin PLP, depicted in Figs. 17(A-F) (SEQ ID NOs:3-8); human MOG, depicted in Fig. 18 (SEQ ID NO:9); rat PLP and variant, depicted in Fig. 19 (SEQ ID NO:10); and rat MAG, depicted in Fig. 20 (SEQ ID NO:11).

5.4 THERAPEUTIC USES

The compositions described in Sections 5.1 through 5.3 may be used to promote nerve regeneration or to prevent or inhibit secondary degeneration which may otherwise follow primary NS injury, e.g., blunt trauma, penetrating trauma, hemorrhagic stroke, ischemic stroke or damages caused by surgery such as tumor excision. In addition, such compositions may be used to ameliorate the effects of disease that result in a degenerative process, e.g., degeneration occurring in either grey or white matter (or both) as a result of various diseases or disorders, including, without limitation: diabetic neuropathy, senile dementias, Alzheimer's disease, Parkinson's Disease, facial nerve (Bell's) palsy, glaucoma, Huntington's chorea, amyotrophic lateral sclerosis (ALS), non-arteritic optic neuropathy, intervertebral disc herniation, vitamin deficiency, prion diseases such as Creutzfeldt-Jakob disease, carpal tunnel syndrome, peripheral neuropathies associated with

various diseases, including but not limited to, uremia, porphyria, hypoglycemia, Sjorgren Larsson syndrome, acute sensory neuropathy, chronic ataxic neuropathy, biliary cirrhosis, primary amyloidosis, obstructive lung diseases, acromegaly, malabsorption syndromes, polycythemia vera, IgA and IgG gammapathies, complications of various drugs (e.g., metronidazole) and toxins (e.g., alcohol or organophosphates), Charcot-Marie-Tooth disease, ataxia telangectasia, Friedreich's ataxia, amyloid polyneuropathies, adrenomyeloneuropathy, Giant axonal neuropathy, Refsum's disease, Fabry's disease, lipoproteinemia, etc.

In a preferred embodiment, the NS-specific activated T cells, the NS-specific antigens, peptides derived therefrom, derivatives thereof or the nucleotides encoding said antigens, or peptides or any combination thereof of the present invention are used to treat diseases or disorders where promotion of nerve regeneration or prevention or inhibition of secondary neural degeneration is indicated, which are not autoimmune diseases or neoplasias. In a preferred embodiment, the compositions of the present invention are administered to a human subject.

While activated NS-specific T cells may have been used in the prior art in the course of treatment to develop tolerance to autoimmune antigens in the treatment of autoimmune diseases, or in the course of immunotherapy in the treatment of NS neoplasms, the present invention can also be used to ameliorate the degenerative process caused by autoimmune diseases or neoplasms as long as it is used in a manner not suggested by such prior art methods. Thus, for example, T cells activated by an autoimmune antigen have been suggested for use to create tolerance to the autoimmune antigen and, thus, ameliorate the autoimmune disease. Such treatment, however, would not have suggested the use of T cells directed to other NS antigens or NS antigens which will not induce tolerance to the autoimmune antigen or T cells which are administered in such a way as to avoid creation of tolerance. Similarly, for neoplasms, the effects of the present invention can be obtained without using immunotherapy processes suggested in the prior art by, for example, using an NS antigen which

does not appear in the neoplasm. T cells activated with such an antigen will still accumulate at the site of neural degeneration and facilitate inhibition of this degeneration, even though it will not serve as immunotherapy for the tumor *per se*.

5.5 FORMULATIONS AND ADMINISTRATION

Pharmaceutical compositions for use in accordance with the present invention may be formulated in conventional manner using one or more physiologically acceptable carriers or excipients. The carrier(s) must be "acceptable" in the sense of being compatible with the other ingredients of the composition and not deleterious to the recipient thereof.

The term "carrier" refers to a diluent, adjuvant, excipient, or vehicle with which the therapeutic is administered. The carriers in the pharmaceutical composition may comprise a binder, such as microcrystalline cellulose, polyvinylpyrrolidone (polyvidone or povidone), gum tragacanth, gelatin, starch, lactose or lactose monochydrate; a disintegrating agent, such as alginic acid, maize starch and the like; a lubricant or surfactant, such as magnesium stearate, or sodium lauryl sulphate; a glidant, such as colloidal silicon dioxide; a sweetening agent, such as sucrose or saccharin; and/or a flavoring agent, such as peppermint, methyl salicylate, or orange flavoring.

Methods of administration include, but are not limited to, parenteral, e.g., intravenous, intraperitoneal, intramuscular, subcutaneous, mucosal (e.g., oral, intranasal, buccal, vaginal, rectal, intraocular), intrathecal, topical and intradermal routes. Administration can be systemic or local.

For oral administration, the pharmaceutical preparation may be in liquid form, for example, solutions, syrups or suspensions, or may be presented as a drug product for reconstitution with water or other suitable vehicle before use. Such liquid preparations may be prepared by conventional means with pharmaceutically acceptable additives such as suspending agents (e.g., sorbitol syrup, cellulose derivatives or hydrogenated edible fats); emulsifying agents (e.g., lecithin or acacia); non-aqueous vehicles (e.g., almond oil,

oily esters, or fractionated vegetable oils); and preservatives (e.g., methyl or propyl-p-hydroxybenzoates or sorbic acid). The pharmaceutical compositions may take the form of, for example, tablets or capsules prepared by conventional means with pharmaceutically acceptable excipients such as binding agents (e.g., pregelatinized maize starch, polyvinyl pyrrolidone or hydroxypropyl methylcellulose); fillers (e.g., lactose, microcrystalline cellulose or calcium hydrogen phosphate); lubricants (e.g., magnesium stearate, talc or silica); disintegrants (e.g., potato starch or sodium starch glycolate); or wetting agents (e.g., sodium lauryl sulphate). The tablets may be coated by methods well-known in the art.

Preparations for oral administration may be suitably formulated to give controlled release of the active compound.

For buccal administration, the compositions may take the form of tablets or lozenges formulated in conventional manner.

The compositions may be formulated for parenteral administration by injection, e.g., by bolus injection or continuous infusion. Formulations for injection may be presented in unit dosage form, e.g., in ampoules or in multidose containers, with an added preservative. The compositions may take such forms as suspensions, solutions or emulsions in oily or aqueous vehicles, and may contain formulatory agents such as suspending, stabilizing and/or dispersing agents. Alternatively, the active ingredient may be in powder form for constitution with a suitable vehicle, e.g., sterile pyrogen free water, before use.

The compositions may also be formulated in rectal compositions such as suppositories or retention enemas, e.g., containing conventional suppository bases such as cocoa butter or other glycerides.

For administration by inhalation, the compositions for use according to the present invention are conveniently delivered in the form of an aerosol spray presentation from pressurized packs or a nebulizer, with the use of a suitable propellant, e.g., dichlorodifluoromethane, trichlorofluoromethane, dichlorotetrafluoroethane, carbon dioxide or other suitable gas. In the case of a pressurized

aerosol the dosage unit may be determined by providing a valve to deliver a metered amount. Capsules and cartridges of, e.g., gelatin, for use in an inhaler or insufflator may be formulated containing a powder mix of the compound and a suitable powder base such as lactose or starch.

In a preferred embodiment, compositions comprising NS-specific activated T cells, an NS-specific antigen or peptide derived therefrom, or derivative thereof, or a nucleotide sequence encoding such antigen or peptide, are formulated in accordance with routine procedures as pharmaceutical compositions adapted for intravenous or intraperitoneal administration to human beings. Typically, compositions for intravenous administration are solutions in sterile isotonic aqueous buffer. Where necessary, the composition may also include a solubilizing agent and a local anesthetic such as lignocaine to ease pain at the site of the injection. Generally, the ingredients are supplied either separately or mixed together. Where the composition is to be administered by infusion, it can be dispensed with an infusion bottle containing sterile pharmaceutical grade water or saline. Where the composition is administered by injection, an ampoule of sterile water or saline for injection can be provided so that the ingredients may be mixed prior to administration.

Pharmaceutical compositions comprising NS-specific antigen or peptide derived therefrom or derivative thereof may optionally be administered with an adjuvant, such as Incomplete Freund's Adjuvant.

The invention also provides a pharmaceutical pack or kit comprising one or more containers filled with one or more of the ingredients of the pharmaceutical compositions of the invention.

In a preferred embodiment, the pharmaceutical compositions of the invention are administered to a mammal, preferably a human, shortly after injury or detection of a degenerative lesion in the NS. The therapeutic methods of the invention may comprise administration of an NS-specific activated T cell or an NS-specific antigen or peptide derived therefrom or derivative thereof, or a nucleotide sequence encoding such antigen or peptide, or any combination thereof.

When using combination therapy, the NS-specific antigen may be administered before, concurrently or after administration of NS-specific activated T cells, a peptide derived from an NS-specific antigen or derivative thereof or a a nucleotide sequence encoding such antigen or peptide.

In one embodiment, the compositions of the invention are administered in combination with one or more of the following (a) mononuclear phagocytes, preferably cultured monocytes (as described in PCT publication No. WO 97/09985, which is incorporated herein by reference in its entirety), that have been stimulated to enhance their capacity to promote neuronal regeneration; (b) a neurotrophic factor such as acidic fibroblast growth factor; and (c) an anti-inflammatory therapeutic substance (i.e., an anti-inflammatory steroid, such as dexamethasone or methylprednisolone, or a non-steroidal anti-inflammatory peptide, such as Thr-Lys-Pro (TKP)).

In another embodiment, mononuclear phagocyte cells according to PCT Publication No. WO 97/09985 and U.S. patent application Serial No. 09/041,280, filed March 11, 1998, are injected into the site of injury or lesion within the CNS, either concurrently, prior to, or following parenteral administration of NS-specific activated T cells, an NS-specific antigen or peptide derived therefrom or derivative thereof, or a nucleotide sequence encoding such antigen or peptide

In another embodiment, administration of NS-specific activated T cells, NS-specific antigen or peptide sequence encoding such antigen or peptide, may be administered as a single dose or may be repeated, preferably at 2 week intervals and then at successively longer intervals once a month, once a quarter, once every six months, etc. The course of treatment may last several months, several years or occasionally also through the life-time of the individual, depending on the condition or disease which is being treated. In the case of a CNS injury, the treatment may range between several days to months or even years, until the condition has stabilized and there is no or only a limited risk of development of secondary degeneration. In chronic human disease or Parkinson's disease, the therapeutic treatment in accordance with the invention may be for life.

As will be evident to those skilled in the art, the therapeutic effect depends at times on the condition or disease to be treated, on the individual's age and health condition, on other physical parameters (e.g. gender, weight, etc.) of the individual, as well as on various other factors, e.g., whether the individual is taking other drugs, etc.

The optimal dose of the therapeutic compositions comprising NS-specific activated T cells of the invention is proportional to the number of nerve fibers affected by NS injury or disease at the site being treated. In a preferred embodiment, the dose ranges from about 5×10^6 to about 10^7 for treating a lesion affecting about 10^5 nerve fibers, such as a complete transection of a rat optic nerve, and ranges from about 10^7 to about 10^8 for treating a lesion affecting about 10^6 -10^7 nerve fibers, such as a complete transection of a human optic nerve. As will be evident to those skilled in the art, the dose of T cells can be scaled up or down in proportion to the number of nerve fibers thought to be affected at the lesion or site of injury being treated.

5.6 ESTABLISHMENT OF AUTOLOGOUS CELL BANKS FOR T LYMPHOCYTES

To minimize secondary damage after nerve injury, patients can be treated by administering autologous or semi-allogeneic T lymphocytes sensitized to at least one appropriate NS antigen. As the window of opportunity has not yet been precisely defined, therapy should be administered as soon as possible after the primary injury to maximize the chances of success, preferably within about one week.

To bridge the gap between the time required for activation and the time needed for treatment, a bank can be established with personal vaults of autologous T lymphocytes prepared for future use for neuroprotective therapy against secondary degeneration in case of NS injury. T lymphocytes are isolated from the blood and then sensitized to a NS antigen. The cells are then frozen and suitably stored under the person's name, identity number, and blood group, in a cell bank until needed.

Additionally, autologous stem cells of the CNS can be processed and stored for potential use by an individual patient in the event of traumatic disorders of the NS such as ischemia or mechanical injury, as well as for treated neurodegenerative conditions such as Alzheimer's disease or Parkinson's disease. Alternatively, semi-allogeneic or allogeneic T cells can be stored frozen in banks for use by any individual who shares one MHC type II molecule with the source of the T cells.

The following examples illustrate certain features of the present invention but are not intended to limit the scope of the present invention.

EXAMPLE: ACCUMULATION OF ACTIVATED T CELLS IN INJURED OPTIC NERVE

6.1 MATERIALS AND METHODS

6.1.1 ANIMALS

Female Lewis rats were supplied by the Animal Breeding Center of the Weizmann Institute of Science (Rehovot, IL), matched for age (8-12 weeks) and housed four to a cage in a light and temperature-controlled room.

6.1.2 MEDIA

The T cell proliferation medium contained the following: Dulbecco's modified Eagle's medium (DMEM, Biological Industries, Israel) supplemented with 2mM L-glutamine (L-Glu, Sigma, USA), 5×10^{-5} M 2-mercaptoethanol (2-ME, Sigma), penicillin (100 IU/ml; Biological Industries), streptomycin (100 μ/ml; Biological Industries), sodium pyruvate (1 mM;. Biological Industries), non-essential amino acids (1 ml/100 ml; Biological Industries) and autologous rat serum 1% (vol/vol) (Mor et al., Clin. Invest. 85:1594, 1990). Propagation medium contained: DMEM, 2-ME, L-Glu, sodium pyruvate, non-essential amino acids and antibiotics in the same concentration as above with the addition of 10% fetal calf serum (FCS), and 10% T cell growth factor (TCGF) obtained from the supernatant of concanavalin A-stimulated spleen cells (Mor et al., *supra*, 1990).

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previously described (Duvdevani et al., Neurol. Neurosci. 2:31-38, 1990). Briefly, rats were deeply anesthetized by i.p. injection of Rompum (xylazine, 10 mg/kg; Vitamed, Israel) and Vetaler (ketamine, 50 mg/kg; Fort Dodge Laboratories, Fort Dodge, Iowa). Using a binocular operating microscope, a lateral canthotomy was performed in the right eye and the conjunctiva was incised lateral to the cornea. After separation of the retractor bulbi muscles, the optic nerve was exposed intraorbitally by blunt dissection. Using calibrated cross-action forceps, a moderate crush injury was inflicted on the optic nerve, 2mm form the eye (Duvdevani et al., Instructure Neurology and Neuroscience 2:31, 1990). The contralateral nerve was left undisturbed and was used as a control.

6.1.6 IMMUNOCYTOCHEMISTRY OF T CELLS

Longitudinal cryostat nerve sections (20 μm thick) were picked up onto gelatin glass slides and frozen until preparation for fluorescent staining. Sections were thawed and fixed in ethanol for 10 minutes at room temperature, washed twice with double-distilled water (ddH_2O), and incubated for 3 minutes in PBS containing 0.05% polyoxyethylene-sorbitan monolaurate (Tween-20; Sigma, USA). Sections were then incubated for 1 hr at room temperature with a mouse monoclonal antibody directed against rat T cell receptor (TCR) (1:100, Hunig et al., J. Exp. Med., 169:73, 1989), in PBS containing 3% FCS and 2% BSA. After three washes with PBS containing 0.05% Tween-20, the sections were incubated with fluorescein isothiocyanate-conjugated goat anti-mouse IgG (with minimal cross-section to rat, human, bovine and horse serum proteins) (Jackson ImmunoResearcch, West Grove, Pennsylvania) for one hour at room temperature. The sections were then washed with PBS containing Tween-20 and treated with glycerol containing 1,4-diazobicyclo-(2,2,2) octane (Sigma), to inhibit quenching of fluorescence. The sections were viewed with a Zeis microscope and cells were counted. Staining in the absence of first antibody was negative.

6.2. RESULTS

Fig. 1 shows accumulation of T cells measured immunohistochemically. The number of T cells was considerably higher in injured nerves rats injected with anti-MBP, anti-OVA or anti-p277 cells; statistical analysis (one-way ANOVA) showed significant differences between T cell numbers in injured optic nerves of rats injected with ant-MBP, anti-OVA, or anti-p277 T cells and in injured optic nerves of rats injected with PBS ($P<0.001$); and between injured optic nerves and uninjured optic nerves of rats injected with anti-MBP, anti-OVA, or anti-p277 T cells ($P<0.001$).

EXAMPLE: NEURPROTECTION BY AUTOIMMUNE ANTI-MBP T CELLS

7.1 MATERIAL AND METHODS

Animals, media, antigens, crush injury of rat optic nerve, sectioning of nerves, T cell lines, and immunolabeling of nerve sections are described in Section 6, *supra*.

7.1.1. RETROGRADE LABELING AND MEASUREMENT OF PRIMARY DAMAGE AND SECONDARY DEGENERATION

Primary damage of the optic nerve axons and their attached retinal ganglion cells (RGCs) were measured after the immediate post-injury application of the fluorescent lipophilic dye 4-(4-(didecylamino)styryl)-N-methylpyridinium iodide (4-Di-Asp) (Molecular Probes Europe BV, Netherlands) distal to the site of injury. Only axons that are intact are capable of transporting the dye back to their cell bodies; therefore, the number of labeled cell bodies is a measure of the number of axons that survived the primary damage. Secondary degeneration was also measured by application of the dye distal to the injury site, but two weeks after the primary lesion was inflicted. Application of the neurotracer dye distal to the site of the primary crush after two weeks ensures that only axons that survived both the primary damage and the secondary degeneration will be counted. This approach makes it possible to differentiate between neurons that are still functionally intact and neurons in which the axons are injured but the cell

bodies are still viable, as only those neurons whose fibers are morphologically intact can take up dye applied distally to the site of injury and transport it to their cell bodies. Using this method, the number of labeled ganglion cells reliably reflects the number of still-functioning neurons. Labeling and measurement were done by exposing the right optic nerve for a second time, again without damaging the retinal blood supply. Complete axotomy was done 1-2 mm from the distal border of the injury site and solid crystals (0.2-0.4 mm in diameter) of 4-Di-10-Asp were deposited at the site of the newly formed axotomy. Uninjured optic nerves were similarly labeled at approximately the same distance from the globe. Five days after dye application, the rats were killed. The retina was detached from the eye, prepared as a flattened whole mount in 4% paraformaldehyde solution and examined for labeled ganglion cells by fluorescence microscopy. The percentage of RGCs surviving secondary degeneration was calculated using the following formula: (Number of spared neurons after secondary degeneration)/(Number of spared neurons after primary damage) x 100.

7.1.2 ELECTROPHYSIOLOGICAL RECORDINGS

Nerves were excised and their compound action potentials (CAPs) were recorded *in vitro* using a suction electrode experimental set-up (Yoles et al., J. Neurotrauma 13:49-57, 1996). At different times after injury and injection of T cells or PBS, rats were killed by intraperitoneal injection of pentobarbitone (170 mg/kg) (CTS Chemical Industries, Israel). Both optic nerves were removed while still attached to the optic chiasma, and were immediately transferred to a vial containing a fresh salt solution consisting of 126 mM NaCl, 3 mM KCl, 1.25 mM NaH_2PO_2 26 mM $NaHCO_3$ 2 mM $MgSO_4$, 2 mM $CaCl_2$ and 10 mM D-glucose, aerated with 95% O_2 and 5% CO_2 at room temperature. After 1 hour, electrophysiological recordings were made. In the injured nerve, recordings were made in a segment distal to the injury site. This segment contains axons of viable retinal ganglion cells that have escaped both primary and secondary damage, as well as the distal stumps of non-viable retinal ganglion cells

that have not yet undergone Wallerian degeneration. The nerve ends were connected to two suction Ag-AgCl electrodes immersed in the bathing solution at 37°C. A stimulating pulse was applied through the electrode, and the CAP was recorded by the distal electrode. A stimulator (SD9; Grass Medical Instruments, Quincy, Massachusetts) was used for supramaximal electrical stimulation at a rate of 1 pps to ensure stimulation of all propagating axons in the nerve. The measured signal was transmitted to a microelectrode AC amplifier (model 1800; A-M Systems, Everett, Washington). The data were processed using the LabView 2.1.1 data acquisition and management system (National Instruments, Austin, Texas). For each nerve, the difference between the peak amplitude and the mean plateau of eight CAPs was computed and was considered as proportional to the number of propagating axons in the optic nerve. The experiments were done by experimentors "blinded", to sample identity. In each experiment the data were normalized relative to the mean CAP of the uninjured nerves from PBS-injected rats,

7.1.3 CLINICAL EVALUATION OF EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS

Clinical disease was scored every 1 to 2 days according to the following neurological scale: 0, no abnormality; 1, tail atony; 2, hind limb paralysis; 3, paralysis extending to thoracic spine; 4, front limb paralysis; 5, moribund state.

7.2 RESULTS

7.2.1 NEUROPROTECTION BY AUTOIMMUNE anti-MBP T CELLS

Morphological analyses were done to assess the effect of the T cells on the response of the nerve to injury, and specifically on secondary degeneration. Rats were injected intraperitoneally immediately after optic nerve injury with PBS or with 1 x 10^7 activated T cells of the various cell lines. The degree of primary damage to the optic nerve axons and their attached RGCs was measured by injecting the dye 4-Di-10-Asp

distal to the site of the lesion immediately after the injury. A time lapse of 2 weeks between a moderate crush injury and dye application is optimal for demonstrating the number of still viable labeled neurons as a measure of secondary degeneration, and as the response of secondary degeneration to treatment. Therefore, secondary degeneration was quantified by injecting the dye immediately or 2 weeks after the primary injury, and calculating the additional loss of RGCs between the first and the second injections of the dye. The percentage of RGCs that had survived secondary degeneration was then calculated. The percentage of labeled RGCs (reflecting still-viable neurons) was significantly greater in the retinas of the rats injected with anti-MBP T cells than in the retinas of the PBS-injected control rats (Fig. 2). In contrast, the percentage of labeled RGCs in the retinas of the rats injected with anti-OVA or anti-p277 T cells was not significantly greater than that in the control retinas. Thus, although the three T cell lines accumulated at the site of injury, only the MBP-specific autoimmune T cells had a substantial effect in limiting the extend of secondary degeneration. Labeled RGCs of injured optic nerves of rats injected with PBS (Fig. 3A), with anti-p277 T cells (Fig. 3B) or with anti-MBP T cells (FIG. 3C) were compared morphologically using micrographs.

7.2.2 CLINICAL SEVERITY OF EAE

Animals were injected i.p. with 10^7 T_{MBP} cells with or without concurrent optic nerve crush injury. The clinical course of the rats injected with the T_{MBP} cells was evaluated according to the neurological paralysis scale. Each group contained 5-9 rats. The functional autoimmunity of the injected anti-MBP T cells was demonstrated by the development of transient EAE in the recipients of these cells. As can be seen in Fig. 4A, the course and severity of the EAE was not affected by the presence of the optic nerve crush injury.

7.2.3 SURVIVAL OF RGCS IN NON-INJURED NERVES

Animals were injected i.p. with 10^7 T_{MBP} cells or PBS. Two weeks later, 4-Di-10-Asp was applied to the optic nerves. After five days the retinal were excised and flat

mounted. Labeled RGCs from five fields (located at approximately the same distance from the optic disk), in each retina were counted and their average number per are (mm^2) was calculated.

As can be seen in Fig. 4B, there is no difference in the number of surviving RGCs per area (mm^2) in non-injured optic nerves of rats injected with anti-MBP T cells compared to in rats injected with PBS.

7.2.4. NEUROPROTECTION BY T CELLS REACTIVE TO A CRYPTIC EPITOPE

To determine whether the neuroprotective effect of the anti-MBP T cells is correlated with their virulence, the effect of T cells reactive to a "cryptic" epitope of MBP, the peptide 51-70 (p51-70) was examined. "Cryptic" epitopes activate specific T cells after an animal is immunized with the particular peptide, but not with the whole antigen (Mor et al., J. Immunol. 155:3693-3699. 1995). The T cell line reactive to the whole MBP and the T cell line reactive to the cryptic epitope p51-70 were compared for the severity of the EAE they induced, and for their effects on secondary degeneration. In rats injected with the T cell line reactive to the cryptic epitope, disease severity (as manifested by the maximal EAE score) was significantly lower than that in rats injected with the T cell line reactive to the whole protein (Table 1). Whereas anti-MBP T cells caused clinical paralysis of the limbs, rats injected with the anti-p51-70 T cells developed only tail atony, not hind limb paralysis, and almost none showed weakness of the hind limbs. Despite this difference in EAE severity, the neuroprotective effect of the less virulent (anti-p51-70) T cells was similar to that of the more virulent (anti-MBP) T cells (Fig. 5). The percentage of RGCs surviving secondary degeneration in the retinas of rats injected with either of the lines was significantly higher than in the retinas of the PBS-injected rats. Thus, there was no correlation between the neuroprotective effect of the autoimmune T cells and their virulence. It is possible that the anti-p51-70 T cells encounter little antigen in the intact CNS, and therefore cause only mild EAE. Their target antigen

may however become more available after injury, enabling these T cells to exert a neuroprotective effect.

TABLE 1. Anti-MBP and anti-p51-70 T cells Vary in Pathogenicity

T Cell Line	Clinical EAE	Mean Max. Score
Whole MBP	Moderate to severe	2.00 + 0.2
p51-70 of MBP	Mild	0.70 + 0.2

Immediately after optic nerve crush injury, Lewis rats were injected with activated anti-MBP T cells or anti-p51-70 T cells. The clinical course of EAE was evaluated according to the neurological paralysis scale. The mean maximal (max.) score ± s.e.m. was calculated as the average maximal score of all the diseased rats in each group. The table is a summary of nine experiments. Each group contains five to ten rats. Statistical analysis showed a significant difference between the mean maximal score of rats injected with anti-MBP T cells and that of rats injected with anti-p51-70 T cells (*P*=0.039, Student's t-test).

7.2.5 ELECTROPHYSIOLOGICAL ACTIVITY

To confirm the neuroprotective effect of the anti-MBP T cells, electrophysiological studies were done. Immediately after optic nerve injury, the rats were injected intraperitoneally with PBS or with 1 x 10^7 activated anti-MBP or anti-OVA T cells. The optic nerves were excised 7, 11 or 14 days later and the compound action potentials (CAPs), a measure of nerve conduction, were recorded from the injured nerves. On day 14, the mean CAP amplitudes of the distal segments recorded from the injured nerves obtained from the PBS-injected control rats were 33% to 50% of those recorded from the rats injected with the anti-MBP T cells (Fig. 6A, Table 2). As the distal segment of the injured nerve contains both neurons that escaped the primary insult and injured neurons that have not yet degenerated, the observed neuroprotective effect could reflect the rescue of spared neurons, or a delay of Wallerian degeneration of the injured neurons (which normally occurs in the distal stump), or both. No effect of the injection anti-MBP T cells on the mean CAP amplitudes of uninjured nerves was

observed (Fig. 6B, Table 2). It is unlikely that the neuroprotective effect observed on day 14 could have been due to the regrowth of nerve fibers, as the time period was too short for this.

The strong neuroprotective effect of the anti-MBP T cells seen on day 14 was associated with a significantly decreased CAP amplitude recorded on day 7 (Table 2). The anti-MBP T cells manifested no substantial effect on the uninjured nerve on day 7, indicating that the reduction in electrophysiological activity observed in the injured nerve on day 7 might reflect the larger number of T cells present at the injury site relative to the uninjured nerve (Fig. 1). The observed reduction in CAP amplitude in the injured nerve on day 7 reflected a transient resting state in the injured nerve. This transient effect has not only disappeared, but was even reversed by day 14 (Table 2). Early signs of the neuroprotective effect could already be detected on day 11 in the rats injected with anti-OVA T cells, no reduction in CAP amplitude on day 7 could be detected in either the injured or the uninjured nerves, and no neuroprotective effect was observed on day 14 (Table 2). Thus, it seems that the early reduction in CAP and the late neuroprotection shown specifically by the anti-MBP T cells are related.

TABLE 2. Transient reduction in electrophysiological activity of the injured optic nerve induced by anti-MBP T cells, followed by a neuroprotective effect

	Uninjured Optic Nerve		Injured Optic Nerve	
	Day 7	Day 14	Day 7	Day 14
Ratio (%) T_{MPB}/PBS	89.9±9.4 (n=22)	101.2±22.7 (n=10)	63.8* ±14.9 (n=17)	243.1**±70.8 (n=8)
Ratio (%) T_{OVA}/PBS	109.7±13.2 (n=11)	92.5±12.6 (n=3)	125.5±24.4 (n=11)	107.3±38.9 (n=4)

Immediately after optic nerve injury, rats were injected with PBS or with activated anti-MBP or anti-OVA T cells. After 7 or 14 days, the CAPs of injured and uninjured nerves were recorded. Ratios were calculated for uninjured nerves as (mean CAP of uninjured nerves from T cell-injected rats/mean CAP of uninjured nerves from PBS-injected rats) x 100, or for injured

nerves as (mean CAP of injured nerves from T cell-injected rats/mean CAP of injured nerves from PBS-injected rats) x 100. The *P* value was calculated by comparing the logarithms of the normalized CAP amplitudes of nerves from PBS-injected rats and rats injected with T cells, using the unpaired Student's test, $^{*}P<0.05$; $^{**}P<0.001$ n=sample size.

7.3 NEUROPROTECTION IN SPINAL CORD INJURY

7.3.1. MATERIALS AND METHODS

Animals, antigens (MBP, OVA) and T cell lines were as described hereinbefore in 6.1.1, 6.1.3 and 6.1.4, respectively

Contusion. Adult rats (300 to 350g) were anesthetized and the spinal cord was exposed by laminectomy at the level of T7-T8. One hour after induction of anesthesia, a 10 gram rod was dropped onto the laminectomized cord from a height of 50 mm. The impactor device (designed by Prof. Wise Young) allowed, for each animal, measurement of the trajectory of the rod and its contact with the spinal cord to allow uniform lesion. Within an hour of the contusion, rats were injected i.p., on a random basis, with either 10^7 cells (specific to either MBP or OVA, depending on the experimental design) or with PBS. Bladder expression was done at least twice a day (particularly during the first 48h after injury, when it was done 3 times a day) until the end of the second week, by which time the rats had developed autonomous bladder voidance. Approximately twice a week, locomotor activity (of the trunk, tail and hind limbs) in an open field was evaluated by placing the rat for 4 min in the middle of a circular enclosure made of molded plastic with a smooth, non-slip floor (90 cm diameter, 7 cm wall height).

7.3.2 RESULTS

The present study of spinal cord neuroprotection was prompted by the previous example that partial injury to an optic nerve can be ameliorated administering T cells directed to a CNS self-antigen. The question was whether autoimmune T cells could have a beneficial effect on recovery from traumatic spinal cord injury with its greater mass of injured CNS tissue and the attendant spinal shock.

Adult Lewis rats were subjected to a calibrated spinal cord contusion produced by dropping a 10 gram weight from a height of 50 mm onto the laminectomized cord at the level of T7-T8 (see description included in Basso et al., Exp-Neurol 139, 244-256, 1996). The rats were then injected intraperitoneally with autoimmune T cells specific to MBP. Control rats were similarly injured but received either no T cells or T cells specific to the non-self antigen ovalbumin (OVA). Recovery of the rats was assessed every 3 to 4 days in terms of their behavior in an open-field locomotion test, in which scores range form 0 (complete paraplegia) to 21 (normal mobility). The locomotor performance of the rats was judged by observers blinded to the identity of the treatment received by the rats. Included in the study was a group of uninjured, sham-operated (laminectomized but not contused) rats which were injected with anti-MBP T cells to verify the activity of the T cells. In all the sham-operated rats, the anti-MBP T cells induced clinical experimental autoimmune encephalomyelitis (EAE), which developed by day 4, reached a peak at day 7 and resolved spontaneously by day 11. Note, therefore, that at the early post-traumatic stage, any effect of the autoimmune T cells on the injured spinal cord, whether positive or negative, would be transiently masked both by spinal shock and by the paralysis of EAE.

Indeed, none of the rats with contused spinal cords showed any locomotor activity in the first few days after the contusion (Fig. 7A). Interestingly, however, the rats treated with anti-MBP T cells recovered earlier from spinal shock; on day 11, for example, when no recovery could be detected in any of the untreated control rats, significant improvement was noted in the T cell-treated rats (Fig. 7A). At all time points thereafter, the rats that had received the autoimmune T cells showed better locomotor recovery than did the untreated injured rats (Fig. 7A). Thus the autoimmune T cells, in spite of being encephalitogenic, did confer significant neuroprotection. Moreover, the phase of neuroprotective activity coincided with the phase of immune paralysis, supporting our suggestion that neuroprotection might be related to transient paralysis.

By one month after trauma the rats in both groups had reached a maximal behavioral score, which then remained at plateau for at least 3 months of follow-up. In the untreated rats, maximal recovery of locomotor behavior, as noted in previous reports of similarly severe contusion (Basso et al., *supra*), was marked by some ineffectual movement of hind-limb joints, but the rats showed no ability support their body weight and walk, and obtained a score of 7.3 ± 0.8 (mean ± SEM). In contrast, the average score of the rats that had been treated with the anti-MBP T cells was 10.2 ± 0.8, and in some rats the value was high as 13. All the rats in the treated group could support their body weight and some could frequently walk in a coordinated fashion. The difference between the two groups, based on 2-factor repeated ANOVA, was statistically significant ($p<0.05$). The recovery curve based on locomotor activity is nonlinear. The above-described increase in motor activity seen after treatment with the anti-MBP T cells could result from much higher percentage of spared tissue based on a linear regression curve on which the behavioral score is correlated with the amount of neural spinal cord tissue (for example, a difference between 11 and 7) on the locomotion score would be read as a difference between 30% and less than 10% of spared tissue).

In another set of experiments the rats were subjected to a more severe insult, resulting in a functional score of 1.9 ± 0.8 (mean ± SEM) in the untreated group and 7.7 ± 1.4 in the treated group (Fig. 7B). This difference of more than 3 fold in behavioral scores was manifested by the almost total lack of motor activity in the control rats as compared with the ability of the autoimmune T cell-treated rats to move all their joints. The beneficial effect was specific to treatment with anti-MBP T cells; no effect was observed after treatment with T cells specific to the non-self antigen OVA (data not shown). The positive effect of the autoimmune T cells seems to be expressed in the preservation of CNS tissue that escaped the initial lesion, i.e., in neuroprotection. Therefore, the magnitude of the effect would be inherently limited by the severity of the insult; the more severe the lesion, the less the amount of spared tissue amenable to neuroprotection.

To determine whether clinical recovery could be explained in terms of preservation of spinal axons, we performed retrograde labeling of the descending spinal tracts by applying the dye rhodamine dextran amine (Brandt et al, J-Neurosci-Methods 45:35-40, 1992) at T12, below the site of damage. The number of dye-stained cells that could be counted in the red nucleus of the brain constituted a quantitative measure of the number of intact axons traversing the area of contusion. Sections of red nuclei from injured rats treated with anti-MBP T cells (Fig. 8) contained 5-fold more labeled cells than sections taken from the untreated injured rats. Photomicrographs of red nuclei taken from rats treated with anti-MBP T cells (with an open field score of 10) and from PBS-treated rats (with a score of 6) are shown in Fig. 8. These findings indicate that the reduction in injury-induced functional deficit observed in the T cell-treated rats can be attributed to the sparing of spinal tracts, resulting in a higher degree of neuron viability.

After a follow-up of more than 3 months, when the locomotor activity scores had reached a plateau, the site of injury of three of PBS-treated animals and three animals treated with anti-MBP T cells were analyzed by diffusion-weighted MRI. The cords were excised in one piece from top to bottom and were immediately placed in fixative (4% paraformaldehyde). Axial sections along the excised contused cord were analyzed. Fig. 9 shows the diffusion anisotropy in axial sections along the contused cord of a rat treated with autoimmune T cells, as compared with that of PBS-treated control rat. The images show anisotropy in the white matter surrounding the grey matter in the center of the cord. Sections taken from the lesion sites of PBS-treated control rats show limited areas of anisotropy, which were significantly smaller than those seen at comparable sites in the cords of the rats treated with the anti-MBP T cells. Quantitative analysis of the anisotropy, reflecting the number of spared fibers, is shown in Fig. 9. The imaging results show unequivocally that, as a result of the treatment with the autoimmune anti-MBP T cells, some spinal cord tracts had escaped the degeneration that would otherwise have occurred.

7.3.3 DISCUSSION OF RESULTS

No cure has yet been found for spinal cord lesions, one of the most common yet devastating traumatic injuries in industrial societies. It has been known for more that 40 years that CNS neurons, unlike neurons of the peripheral nervous system, possess only a limited ability to regenerate after injury. During the last two decades, attempts to promote regeneration have yielded approaches that lead to partial recovery. In the last few years it has become apparent that, although most of the traumatic injuries sustained by the human spinal cord are partial, the resulting functional loss is nevertheless far worse than could be accounted for by the severity of the initial insult; the self-propagating process of secondary degeneration appears to be decisive.

A substantial research effort has recently been directed to arresting injury-induced secondary degeneration. All attempts up to now have been pharmacologically based, and some have resulted in improved recovery from spinal shock. The present study, in contrast, describes a cell therapy that augments what seems to be a natural mechanism of self-maintenance and leads, after a single treatment, to long-lasting recovery. The extent of this recovery appears to exceed that reported using pharmacological methods.

In most tissues, injury-induced damage triggers a cellular immune response that acts to protect the tissue and preserve its homeostasis. This response has been attributed to macrophages and other cells comprising the innate arm of the immune system. Lymphocytes, which are responsible for adaptive immunity, have not been thought to participate in tissue maintenance. Adaptive immunity, according to traditional teaching, is directed against foreign dangers. Our studies now show, however, that the adaptive T cell immune response can be protective even when there is no invasion by foreign pathogens. In the case of tissue maintenance, the specificity of the T cells is to tissue self-antigens.

Our observation of post-traumatic CNS maintenance by autoimmune T cells suggests that we might do well to reevaluate some basic concepts of autoimmunity. T cells that are specific

to CNS self antigens in general, and to MBP in particular, have long been considered to be only detrimental to health. In the present study, however, the same T cell preparation that can produce EAE in the undamaged CNS was found to be neuroprotective in the damaged spinal cord, suggesting that the context of the tissue plays an important part in determining the outcome of its interaction with T cells. It would seem that the tissue deploys specific signals to elicit particular T cell behaviors. Among such signals are costimulatory molecules, particularly members of the B7 family (Lenchow et al., Annu. Rev. Immunol. 14:233-258, 1996). As shown hereinafter, the injured rat optic nerve transiently expresses elevated levels of the costimulatory molecule B7.2, which is constitutively expressed at low levels in the rat CNS white matter and which is thought to be associated with regulation of the cytokine profile of the responding T cells (H. L. Weiner, Annu. Rev. Med. 48:341-51, 1997). The early post-injury availability of the exogenous anti-MBP T cells, coinciding with the observed early post-injury increase in B7.2 would support the idea that signals expressed by the tissue might modulate the T cell response. It is thus conceivable that anti-MBP T cells which cause a monophasic autoimmune disease upon interacting with a healthy CNS nerve, might implement a maintenance program when they interact with damaged CNS tissue expressing increased amounts of B7.2 and probably other costimulatory molecules. The neuroprotective effects of the T cells may be mediated, at least in part, by antigen-dependent regulation of specific cytokines or neurotrophic factors (M. Kerschensteiner et al., J. Exp. Med. 189:865-870, 1999) produced locally at the site of injury.

Thus, the present invention is also directed to manipulating B7.2 co-stimulatory molecule to prevent or inhibit neuronal degeneration and ameliorate the effects of injury to or disease of the nervous system. B7.2 molecule can be up-regulated for this purpose, using drugs or by genetic manipulation, without undue experimentation.

In a recent study, it was reported that injury to the spinal cord triggers a transient autoimmune response to MBP (Popovich et al., J. Neurosci. Res. 45:349-63, 1996). However,

whether that response is detrimental or beneficial remained an open question (Popovich et al, J. Comp. Neurol. 377:443-464, 1997). From our present data, it would appear that the activation of anti-MBP T cells could indeed be beneficial. However, a supplement of exogenous autoimmune T cells may be required to overcome the restrictions on immune reactivity imposed by the immune-privilege of the CNS (J. W. Streilein, Science 270:1158-1159, 1995). The finding that autoimmune response can be advantageous suggests that natural autoimmune T cells may have undergone positive selection during ontogeny, as proposed by the theory of the immunological homunculus (I. R. Cohen, Immunol. Today 13, 490-494 (1992), and are not merely a default resulting from the escape from negative selection of T cells that recognize self antigens (C. A. Janeway, Jr., Immunol. Today 13:11-6, 1992). Such a response could then be considered as a mechanism of potential physiological CNS self-maintenance, which is, however, not sufficient for the purpose because of the immune-privileged character of the CNS.

A single injection of autoimmune T cells lasted for at least 100 days. Thus, this procedure offers a form of self-maintenance. This specific autoimmune response, when properly controlled, is useful as part of a self-derived remedy for spinal cord injury.

EXAMPLE: NEUROPROTECTIVE EFFECTS OF NS-SPECIFIC ANTIGEN

8.1 MATERIALS AND METHODS

Animals, crush injury of rat optic nerve, and retrograde labeling are described above in Sections 6 and 7. A peptide based on amino acids 35-55 of myelin/oligodendrocyte glycoprotein (MOG p35-55) was chemically synthesized at the Weizmann Institute, Israel.

8.1.1 INHIBITION OF SECONDARY DEGENERATION

Rats were injected intradermally in the footpads with MOG p35-55 (50 μg/animal) and IFA, or PBS ten days prior to optic nerve crush injury. Retinal ganglion cells were assessed two weeks after injury using retrograde labeling as described above. The number of RGCs in rats injected with PBS or MOG

p35-55 was expressed as a percentage of the total number of neurons in rats injected with MOG p35-55 in the absence of crush injury.

8.2 RESULTS

As shown in Fig. 10, the number of labeled retinal ganglion cells (indicating viable axons) was about 12.5 fold greater in animals injected with MOG p35-55 compared to animals receiving PBS.

EXAMPLE: NEUROPROTECTIVE EFFECTS OF MBP ADMINISTERED ORALLY

9.1 MATERIALS AND METHODS

Animals, crush injury of rat optic nerve, and retrograde labeling of RGCs are described above in Sections 6 and 7.

9.1.1 INHIBITION OF SECONDARY DEGENERATION

Bovine MBP (Sigma, Israel) (1 mg/dose) was administered to rats by gavage using a blunt needle. MBP was administered 5 times, every third day, beginning 2 weeks prior to optic nerve crush injury. The number of RGCs in treated animals was expressed as a percentage of the total number of neurons in animals subjected to optic nerve crush injury but which did not receive MBP.

9.2 RESULTS

As shown in Fig. 11, the number of labeled RGCs was about 1.3 fold greater in animals treated with MBP compared to untreated animals.

9.3 THE B7.2 COSTIMULATORY MOLECULE IS ASSOCIATED WITH POST-TRAUMATIC MAINTENANCE OF THE OPTIC NERVE BY ORAL ADMINISTRATION OF MBP

9.3.1 INTRODUCTION

Autoimmune T cells can under under certain conditions be beneficial to traumatized CNS axons. The effect of such T cells on the damaged tissue might be influenced by the nature

and amount of the costimulatory molecules it expresses. We show that the B7.2 costimulatory molecule is constitutively expressed in the intact rat optic nerve, and after injury is up-regulated at the margins of the injury site. Pre-injury induction of oral tolerance to MBP resulted in a further post-injury increase in B7.2 at the margins and at the injury site itself, as well as a better preservation of the traumatized nerve. Thus, B7.2 expression in the brain and its up-regulated after trauma seem to be directly related to post-traumatic maintenance displayed by autoimmune T cells.

Neuronal injury in the CNS causes degeneration of directly damaged fibers as well as of fibers that escaped the primary insult. It also triggers a systemic response of autoimmune T cells to MBP, that might affect the course of degeneration of the injured nerve. Whether the effect of these T cells on the nerve is detrimental or beneficial may depend, in part, on the nature and level of the costimulatory molecules expressed by the damaged tissue. Several costimulatory molecules have recently been identified, including the B7 and CD40 molecules (Caux et al., "Activation of Human Dendritic Cells Through CD40 Cross-Linking", J. Exp. Med. 180:1263-1272, 1994; and Lenschow et al., "CD28/B7 System of T Cell Costimulation", Annu. Rev. Immunol. 14:233-258, 1996). CD40 appears to be dominant during cell differentiation in the lymph nodes and B7 during activation of T cells in the target organ (Grewal et al., "Requirement for CD40 Ligand in Costimulation Induction, T Cell Activation, and Experimental Allergic Encephalomyelitis", Science 273:1864-1867, 1996). B7 costimulatory molecules are expressed on antigen-presenting cells (APCs) as B7.1 or B7.2., which might preferentially support activation of the Th1 or the Th2 type of immune response, respectively (Kuchroo et al., "B7-1 and B7-2 costimulatory molecules activate differentially the Th1/Th2 developmental pathways: application to autoimmune disease therapy", Cell 80:707-718, 1995; and Karandikar et al., "Targeting the B7/CD28:CTLA-4 costimulatory system in CNS autoimmune disease", J. Neuroimmunol. 89:10-18, 1998). We were therefor interested in determining the identity B7 subtype expressed in intact and injured CNS white matter, and its

possible influence on the course of the response to the injury.

9.3.2 RESULTS

The costimulatory molecule expressed constitutively in the intact optic nerves of adult Lewis rats was identified as B7.2. (Figs. 12A, 12B). To examine the effects of neurotrauma on the expression of B7 costimulatory molecules, we inflicted a mild crush injury on the optic nerves of Lewis rats and assessed the neural expression of B7 by immunohistochemical analysis. The most striking effect of the injury was seen on B7.2 expression manifested on post-injury day 3 by its elevation at the margins of the injury site (Figs. 12C,D,E). In contrast, expression of B7.1 was not detected in the optic nerve either before or 3 days after injury. On day 7, however, B7.1 was detectable at the site of injury, having pattern reminiscent of that seen for macrophages or microglia (Fig. 12F).

Next, we attempted to determine whether the degenerative response to optic nerve injury could be modified by peripheral manipulation of the immune system. The manipulation chosen was induction of oral tolerance, known to cause a "bystander" T cell immunosuppressive effect (Weiner et al., "Tolerance Immune Mechanisms and Treatment of Autoimmune Diseases", Immunol. Today 18:335-343, 1997). Ingestion of low doses of MBP results in the activation of T cells which, based on antigen recognition, secrete TGF as the dominant cytokine and thus favor an immune response of Th2/3 type (Chen, Y., "Regulatory T Cell Clones Induced by Oral Tolerance: Suppression of Autoimmune Encephalomyelitis", Science 265: 1237-1240, 1994).

Lewis rats were fed with food to which 1 mg of bovine MBP had been added five times daily every other day. Ten days after first receiving the supplement, the rats were subjected to mild unilateral optic nerve crush injury. This time interval between initiation of oral tolerance and injury was chosen to allow adequate build-up of the systemic T cell response. As shown in Fig. 13A and B, the numbers of macrophages or active microglia (indicated by ED-1 labeling)

and T cells (indicated by immunolabeling for T cell receptor), assessed 3 days after injury, did not differ from those observed in control injured rats which did receive any treatment or were fed with PBS. In the rats with induced oral tolerance to MBP, however, the amounts B7.2 were further increased at the margins of the site of injury (Fig. 13C) as compared with controls (Fig. 12E). In addition, B7.2 in the rats with induced oral tolerance to MBP was also elevated at the site of injury relative to the control nerves (Fig. 13C). It seems reasonable to assume that the T cells exposed to MBP via intestinal absorption, upon invading the injured CNS, contributed to the increase in expression of B7.2 by the injured nerve.

We then attempted to determine whether the observed changes in B7.2 expression in the injured rats was correlated with the extent of neuronal degeneration. Acute injury of the rat optic nerve is followed by a process of nerve degeneration, which can be quantified by retrograde labeling of the surviving neurons and counting of the corresponding cell bodies. Two weeks after optic nerve injury the number of surviving retinal ganglion cells (RGCs), representing still-viable neurons, in the group of MBP-fed rats was significantly higher that that in the control group, or than in the group of rats with injured nerves that were fed with ovalbumin. Interestingly, the benefit of the induced oral tolerance to MBP was increased by feeding the rats with more intensive schedule (Fig. 14).

DISCUSSION OF EXPERIMENTAL RESULTS

The results of the experiments described in Sections 6 and 7 show that activated T cells accumulate at a site of injury in the CNS. Furthermore, the results also demonstrate that the accumulation of T cells at the site of injury is a non-specific process, i.e., T cells which accumulated at the site of injury included both T cells which are activated by exposure to an antigen present at the site of injury as well as T cells which are activated by an antigen not normally present in the individual.

The results of experiments described in Section 7 demonstrate that the beneficial effects of T cells in

ameliorating damage due to injury in the CNS are associated with an NS-specific self-antigen as illustrated by MBP. More specifically, the administration of non-recombinant T cells which were activated by exposure to an antigen which can cause autoimmune disease (T_{MBP}), rather than aggravating the injury, led to a significant degree of protection from secondary degeneration. Thus, activating T cells by exposure to a fragment of an NS-specific antigen was beneficial in limiting the spread of injury in the CNS. The present findings show that secondary degeneration can be inhibited by the transfer into the individual on non-recombinant T cells which recognize an NS-specific self antigen which is present at a site of injury. The T cells may recognize cryptic or non-pathogenic epitopes of NS-self antigens.

In addition, the studies described in Sections and 9 show that activation of T cells by administering an immunogenic antigen (e.g. MBP) or immunogenic epitope of an antigen (e.g. MOG p35-55), may be used for preventing or inhibiting secondary CNS degeneration following injury.

The foregoing description of the specific embodiments will so fully reveal the general nature of the invention that others can, by applying current knowledge, readily modify and/or adapt for various applications such specific embodiments without undue experimentation and without departing from the generic concept, and, therefore, such adaptations and modifications should and are intended to be comprehended within the meaning and range of equivalents of the disclosed embodiments. It is to be understood that the phraseology or terminology employed herein is for the purpose of description and not of limitation. The means, materials, and steps for carrying out various disclosed functions may take a variety of alternative forms without departing from the invention. Thus the expressions "means to..." and "means for...", or any method step language, as may be found in the specification above and/or in the claims below, followed by a functional statement, are intended to define and cover whatever structural, physical, chemical or electrical element or structure, or whatever method step, which may now or in the future exist which carries out the recited function, whether or not precisely equivalent to

the embodiment or embodiments disclosed in the specification above, i.e., other means or steps for carrying out the same function can be used; and it is intended that such expressions be given their broadest interpretation.

All publications cited herein are incorporated by reference in their entirety.

WHAT IS CLAIMED IS:

1. A composition for preventing or inhibiting degeneration in the central nervous system or peripheral nervous system for ameliorating the effects injury or disease, comprising:

(a) NS-specific activated T cells;

(b) NS-specific antigen;

(c) a peptide derived from an NS-specific antigen;

(d) a nucleotide sequence encoding an NS-specific antigen;

(e) a nucleotide sequence encoding a peptide derived from an NS-specific antigen, or

(f) any combination of (a)-(e).

2. A composition according to claim 1, for promoting nerve regeneration in the central nervous system or peripheral nervous system for ameliorating the effects of injury or disease.

3. The composition of claim 1 or 2 in which said injury comprises spinal cord injury, blunt trauma, penetrating trauma, hemorrhagic stroke, or ischemic stroke.

4. The composition of claim 1 or 2 in which said disease is Diabetic neuropathy, senile dementia, Alzheimer's disease, Parkinson's Disease, facial nerve (Bell's) palsy, glaucoma, Huntington's chorea, amyotrophic lateral sclerosis, non-arteritic optic neuropathy, or vitamin deficiency.

5. The composition of claim 1 or 2 in which said disease is not an autoimmune disease or a neoplasm.

6. The composition according to any of of claims 1-5 wherein said NS-specific activate T cells of (a) are autologous T cells, or allogeneic T cells from related donors, OR HLA-matched or partially matched, semi-allogeneic or fully allogeneic donors.

7. The composition according to claim 6 wherein said autologous T cells have been stored or are derived from autologous CNS cells.

8. The composition according to claim 6 wherein said T cells are semi-allogeneic T cells.

9. The composition according to any of claims 1-5 wherein said NS-specific antigen of (b) is elected from myelin basic protein (MBP), myelin oligodenrocyte glycoprotein (MOG), proteolipid protein (PLP), myelin-associated glyycoprotein (MAG), S-100, β-amyloid, Thy-1, P0, P2 and neurotransmitter receptors.

10. The composition according to any one of claims 1-5 wherein said peptide derived from an NS-specific antigen is an immunogenic epitope or a cryptic epitope of said antigen.

11. The composition according to claim 10 wherein said peptide is an immunogenic epitope or a cryptic epitope derived from MBP.

12. The composition according to claim 11 wherein said peptide corresponds to the sequences p11, p51-70, p91-110, p131-150, or p151-170 of MBP.

13. The compositions according to any one of claims 1-5 and 11-12 in which said NS-specific antigen or a peptide derived therefrom is administered intravenously, orally, intranasally, intrathecally, intramuscularlly, intradermally, topically, subcutaneously, mucosally (e.g., orally, intranasally, vaginally, rectally) or bucally.

14. The composition according to claim 13 comprising MBP for oral administration.

15. Use of:

(a) NS-specific activated T cells;

(b) an NS-specific antigen;

(c) a peptide derived from an NS-specific antigen;

(d) a nucleotide sequence encoding an NS-specific antigen;

(e) a nucleotide sequence encoding a peptide derived from an NS-specific antigen, or

(f) any combination of (a)-(e),

for the preparation of a composition for preventing or inhibiting neuronal degeneration in the central nervous system or peripheral nervous system for ameliorating the effects of injury or disease.

16. A method for preventing or inhibiting neuronal degeneration in the central nervous system or peripheral

nervous system, which comprises administering to an individual in need thereof an effective amount of:

(a) NS-specific activated T cells;

(b) NS-specific antigen;

(c) a peptide derived from an NS-specific antigen;

(d) a nucleotide sequence encoding an NS-specific antigen;

(e) a nucleotide sequence encoding a peptide derived from an NS-specific antigen, or

(f) any combination of (a)-(e).

17. A method for preventing or inhibiting neuronal degeneration in the central nervous system or peripheral nervous system comprising administering to an individual in need thereof an effective amount of a composition according to any one of claims 1-13 and actively immunizing said individual to build up a critical T cell response.

18. A method for preventing or inhibiting neuronal degeneration in the central nervous system or peripheral nervous system comprising administering to an individual in need thereof an effective amount of a composition for up-regulating B7.2 costimulatory molecule or genetically manipulating B7.2 costimulatory molecule in said individual.

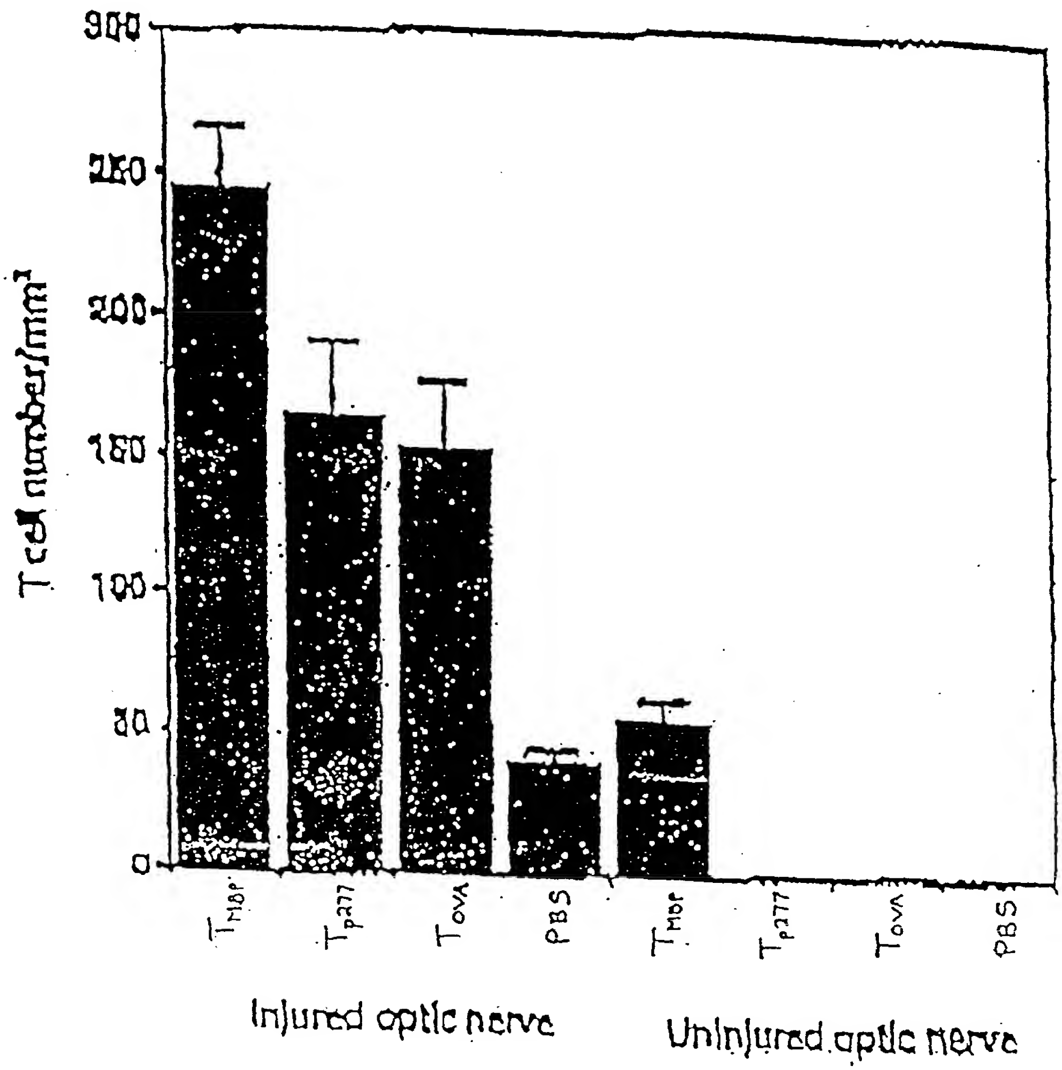

T cell number/mm²
300
250
200
150
100
50
0
T_MBP
T_p277
T_OVA
PBS
T_MBP
T_p277
T_OVA
PBS
Injured optic nerve
Uninjured optic nerve

FIG. 1

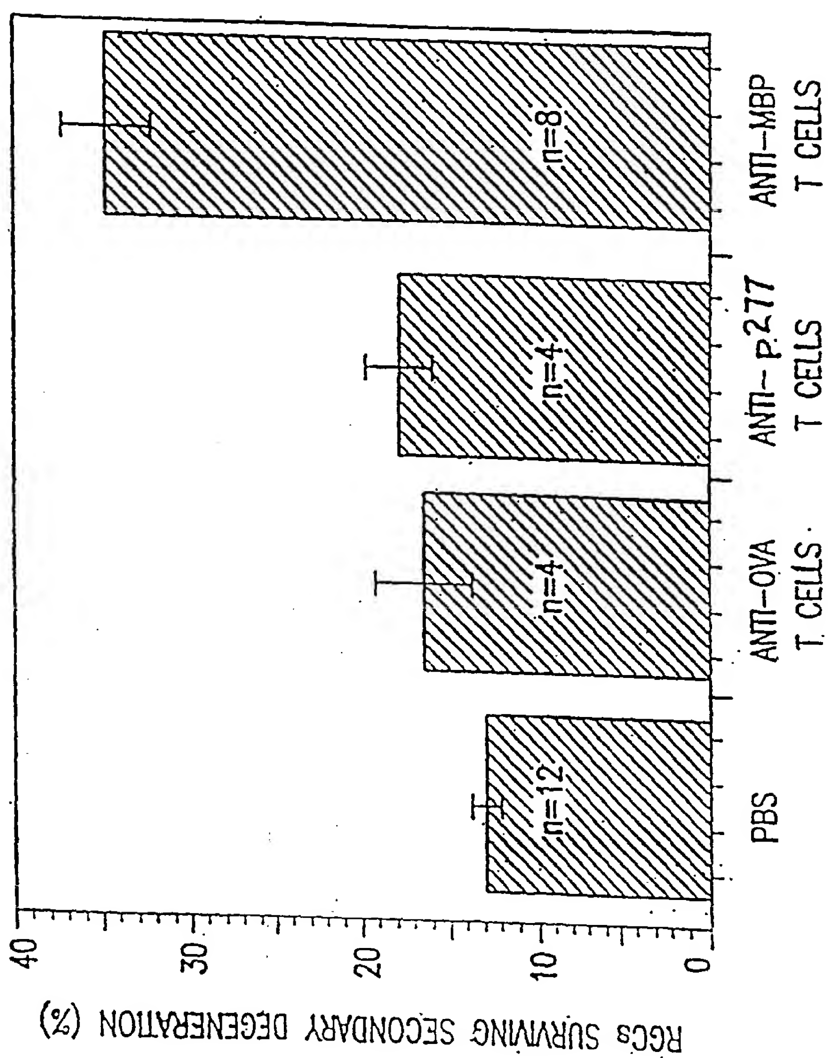

RGCs SURVIVING SECONDARY DEGENERATION (%)
0
10
20
30
40
n=12
n=4
n=4
n=8
PBS
ANTI-OVA T CELLS
ANTI- p277 T CELLS
ANTI-MBP T CELLS

FIG. 2

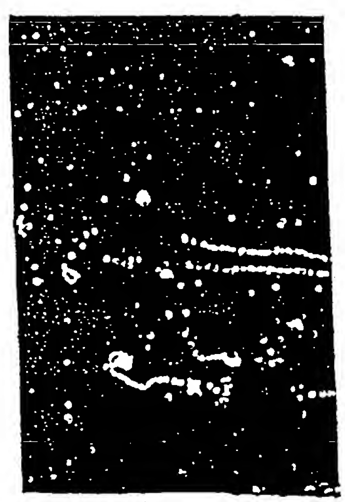


FIG. 3A

FIG. 3B

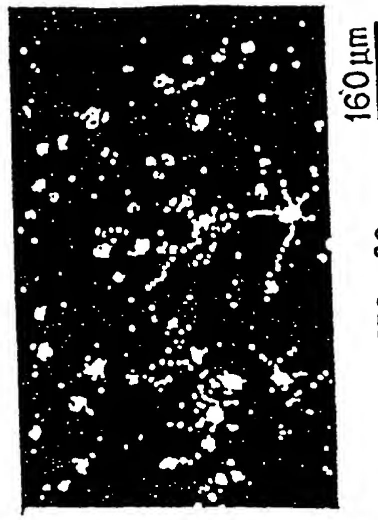


FIG. 3C

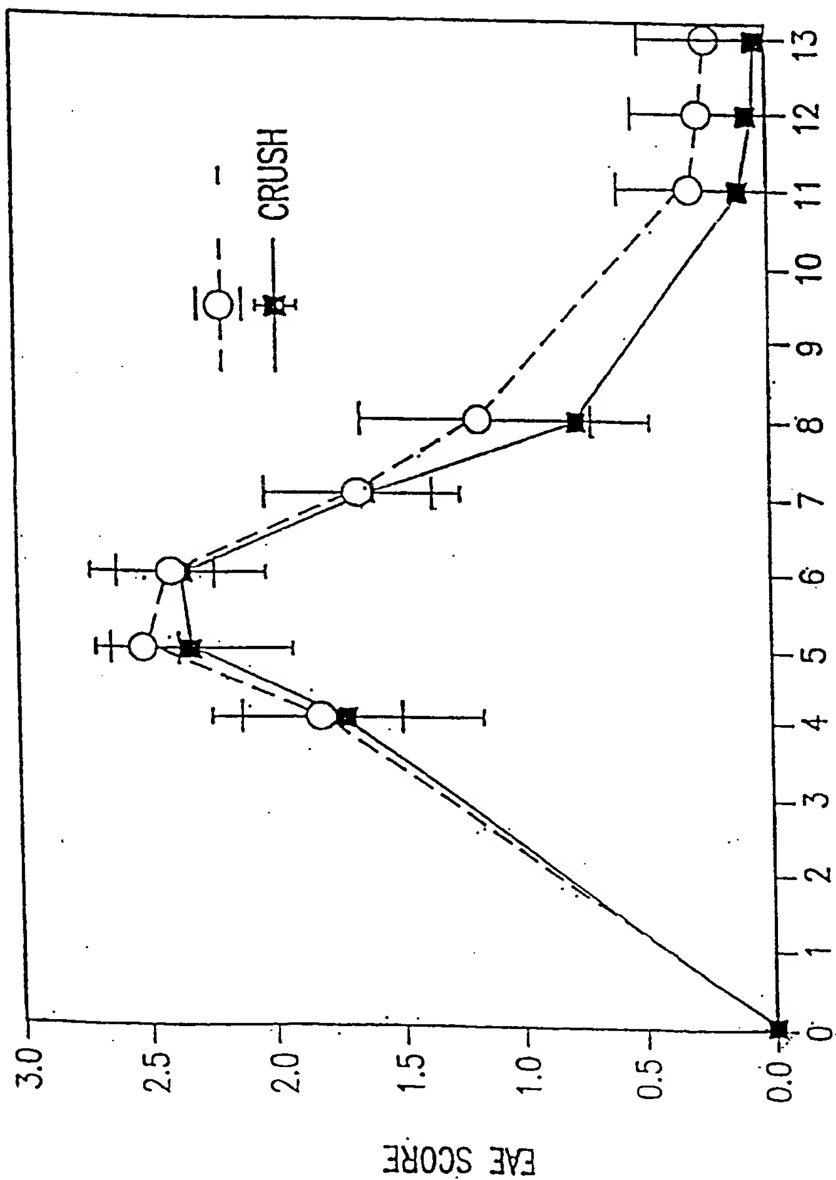


FIG. 4A

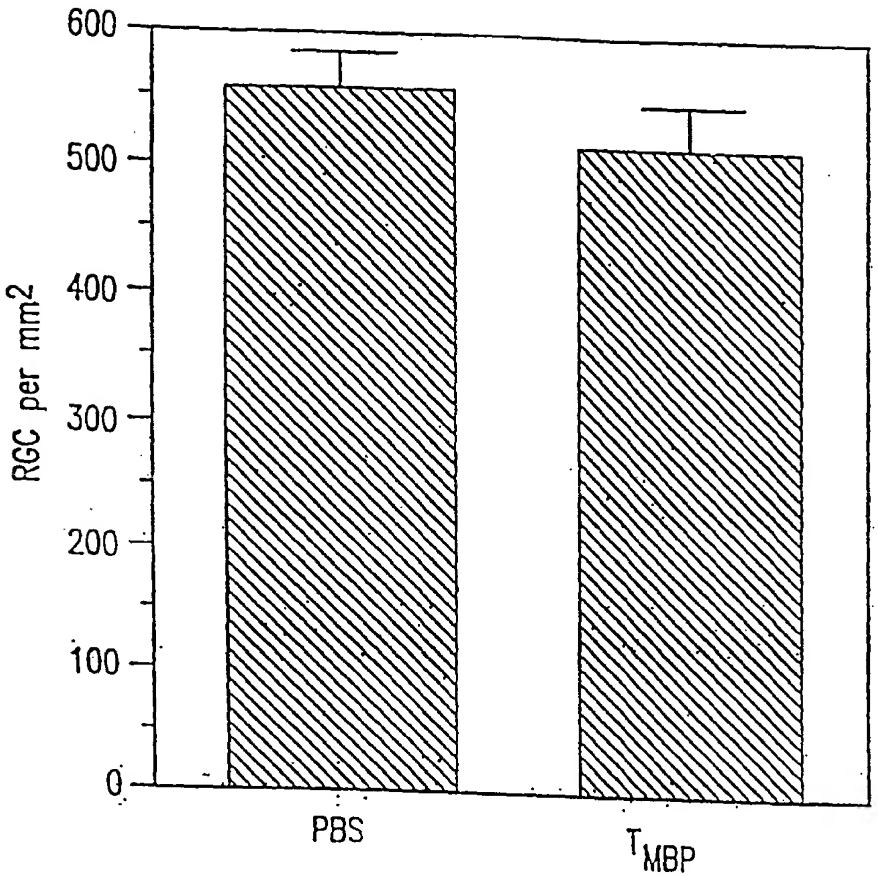


FIG. 4B

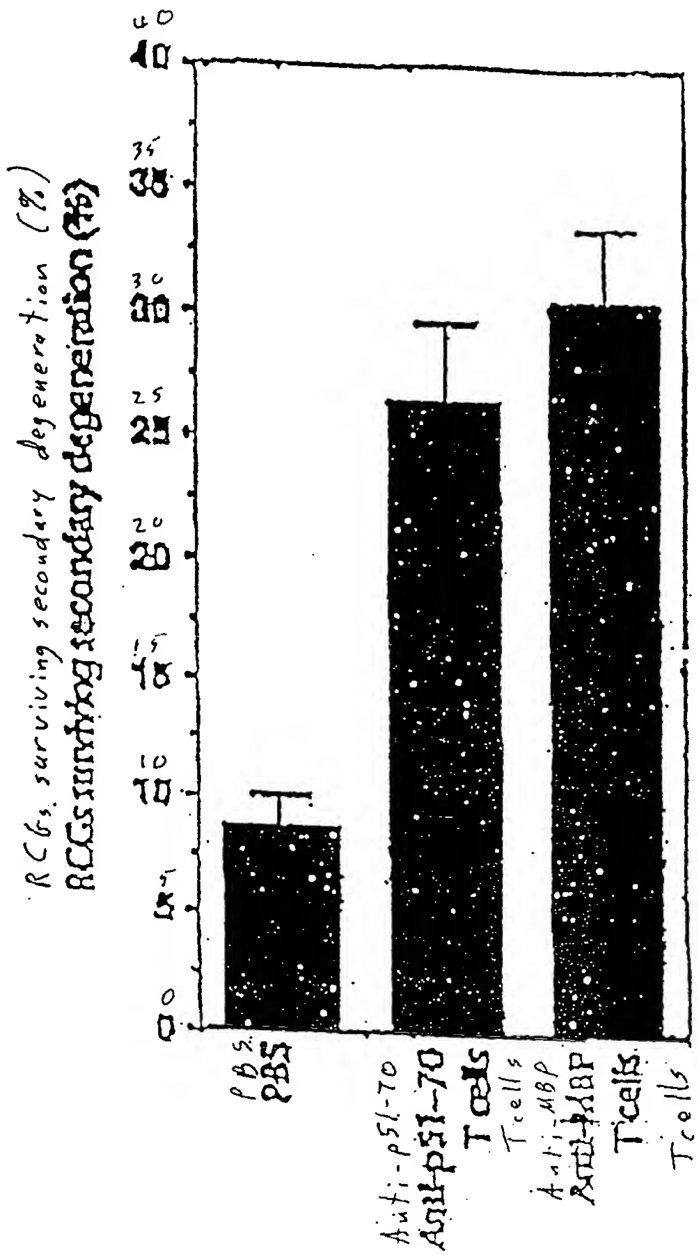

RGCs surviving secondary degeneration (%)
0
5
10
15
20
25
30
35
40
PBS
Anti-p51-70 T cells
Anti-MBP T cells

FIG. 5

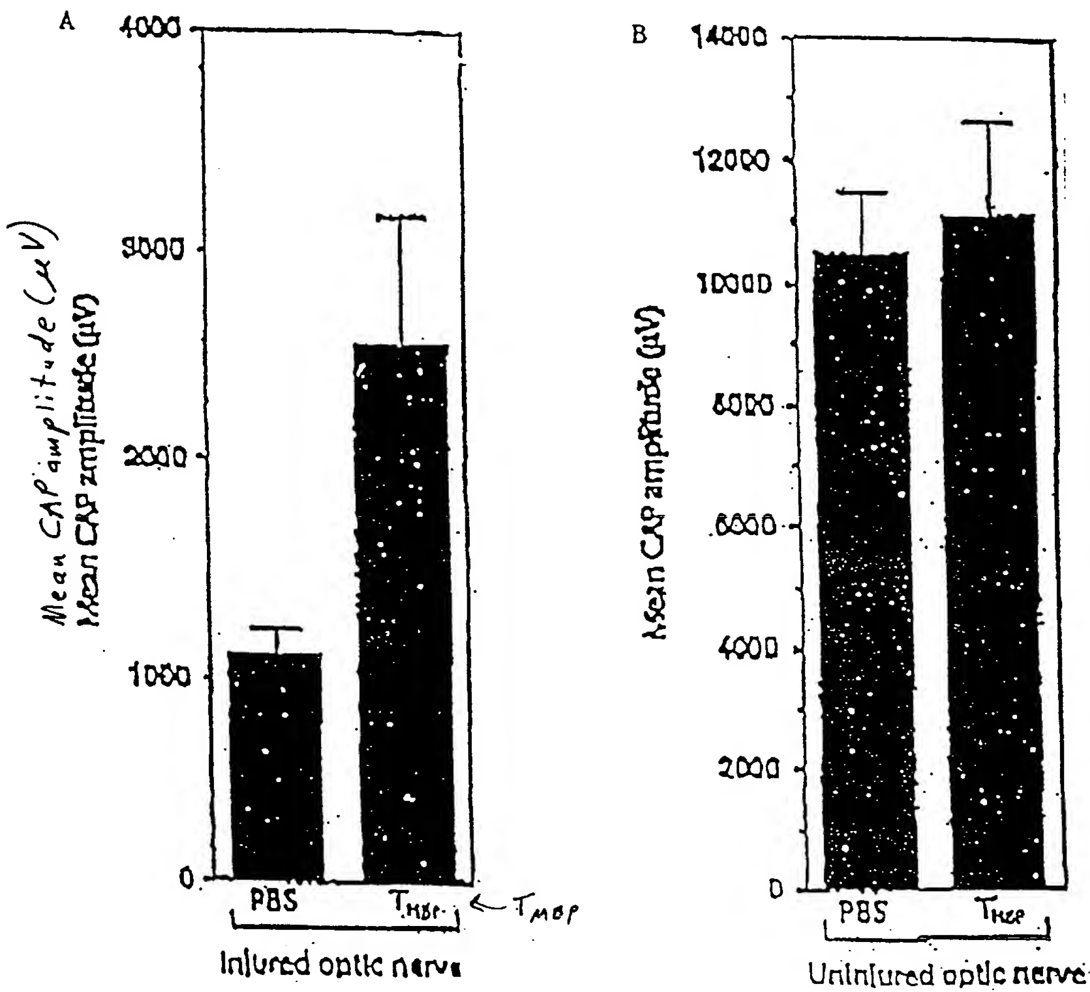

A
4000
3000
2000
1000
0
Mean CAP amplitude (μV)
Mean CAP amplitude (μV)
PBS
T_{MBP}
← T_{MBP}
Injured optic nerve
B
14000
12000
10000
8000
6000
4000
2000
0
Mean CAP amplitude (μV)
PBS
T_{MBP}
Uninjured optic nerve

FIG. 6

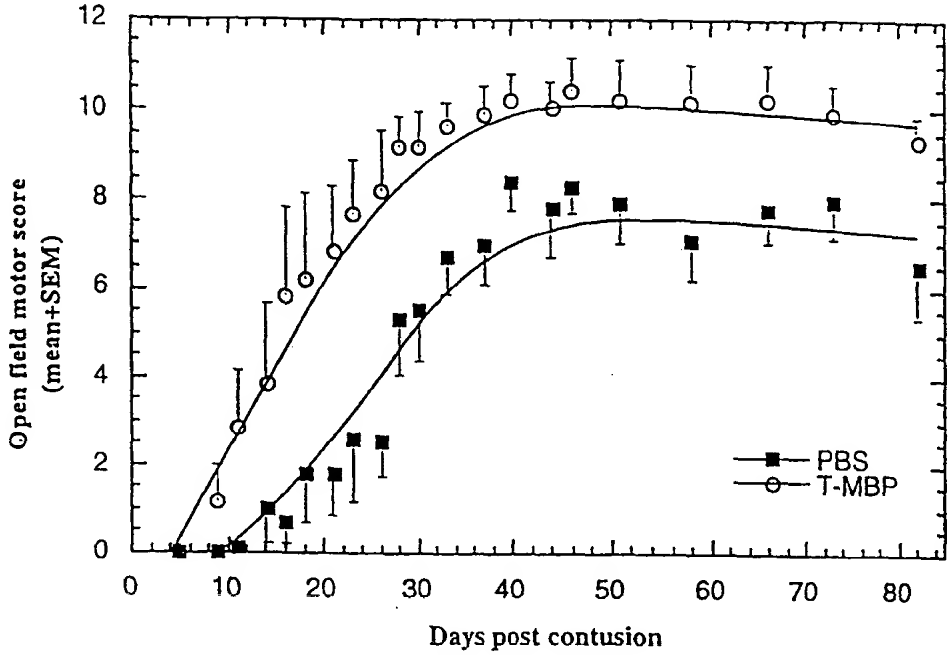

Open field motor score
(mean+SEM)
Days post contusion
PBS
T-MBP
0
2
4
6
8
10
12
0 10 20 30 40 50 60 70 80

FIG. 7A

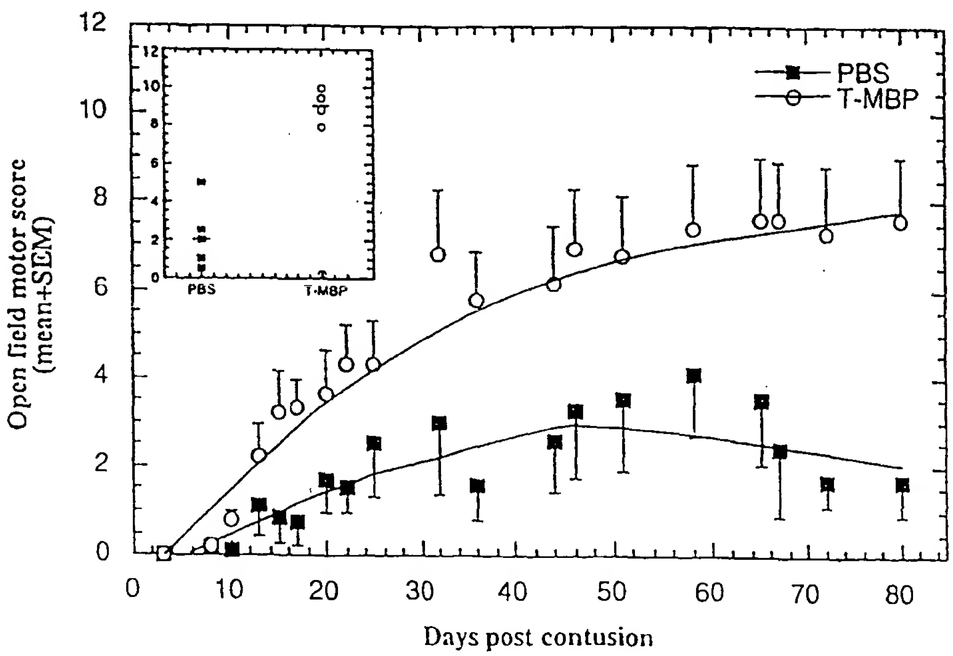
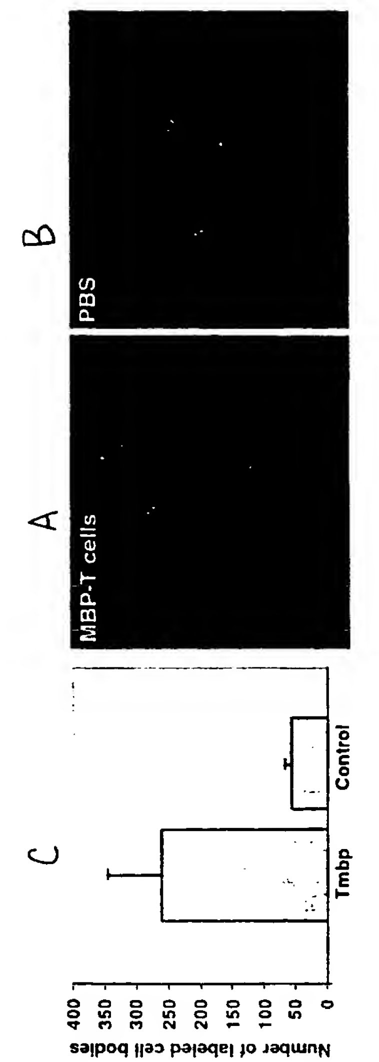

Open field motor score
(mean+SEM)
Days post contusion
PBS
T-MBP
0
2
4
6
8
10
12
0 10 20 30 40 50 60 70 80

FIG. 7B

FIG. 8


A
MBP-T cells
B
PBS
C
Number of labeled cell bodies
400
350
300
250
200
150
100
50
0
Tmbp
Control

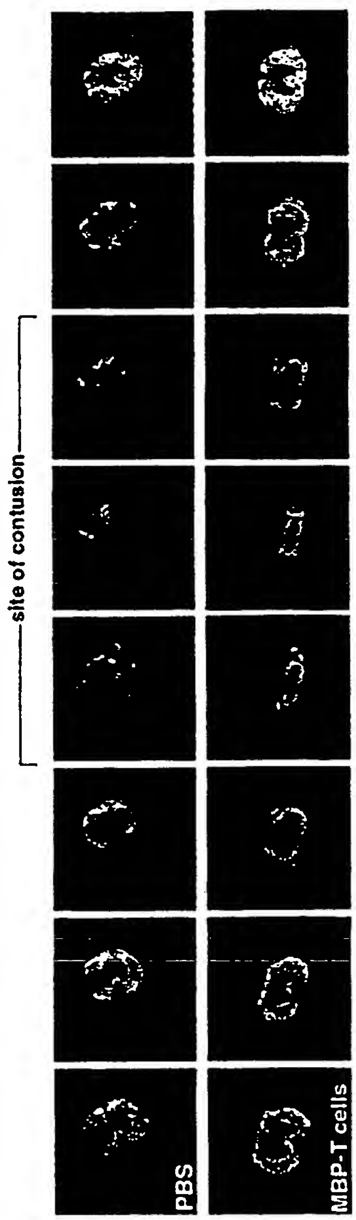


FIG. 9

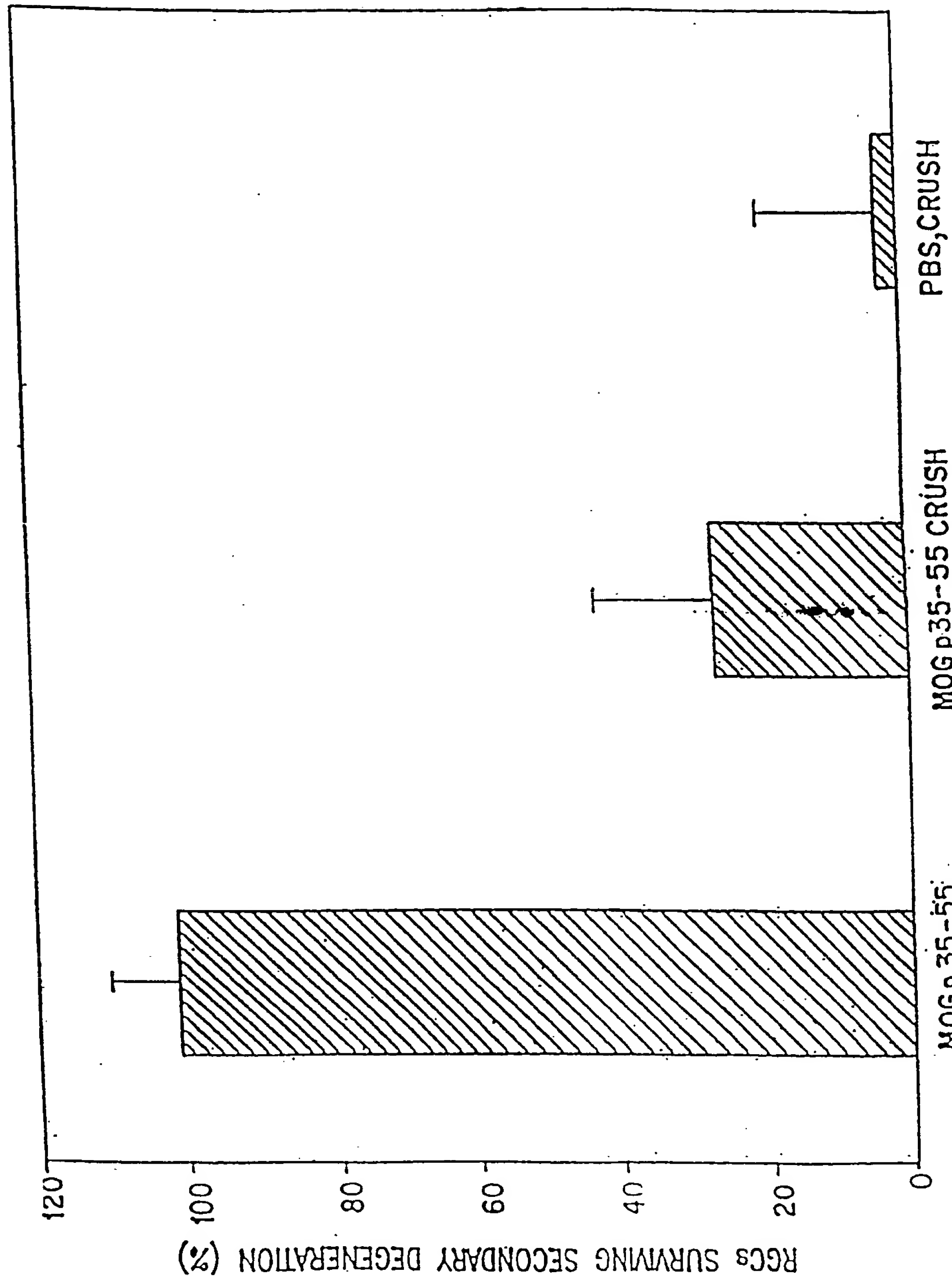

RGCs SURVIVING SECONDARY DEGENERATION (%)
120
100
80
60
40
20
0
MOGp 35-55
MOGp35-55 CRUSH
PBS,CRUSH

FIG. 10

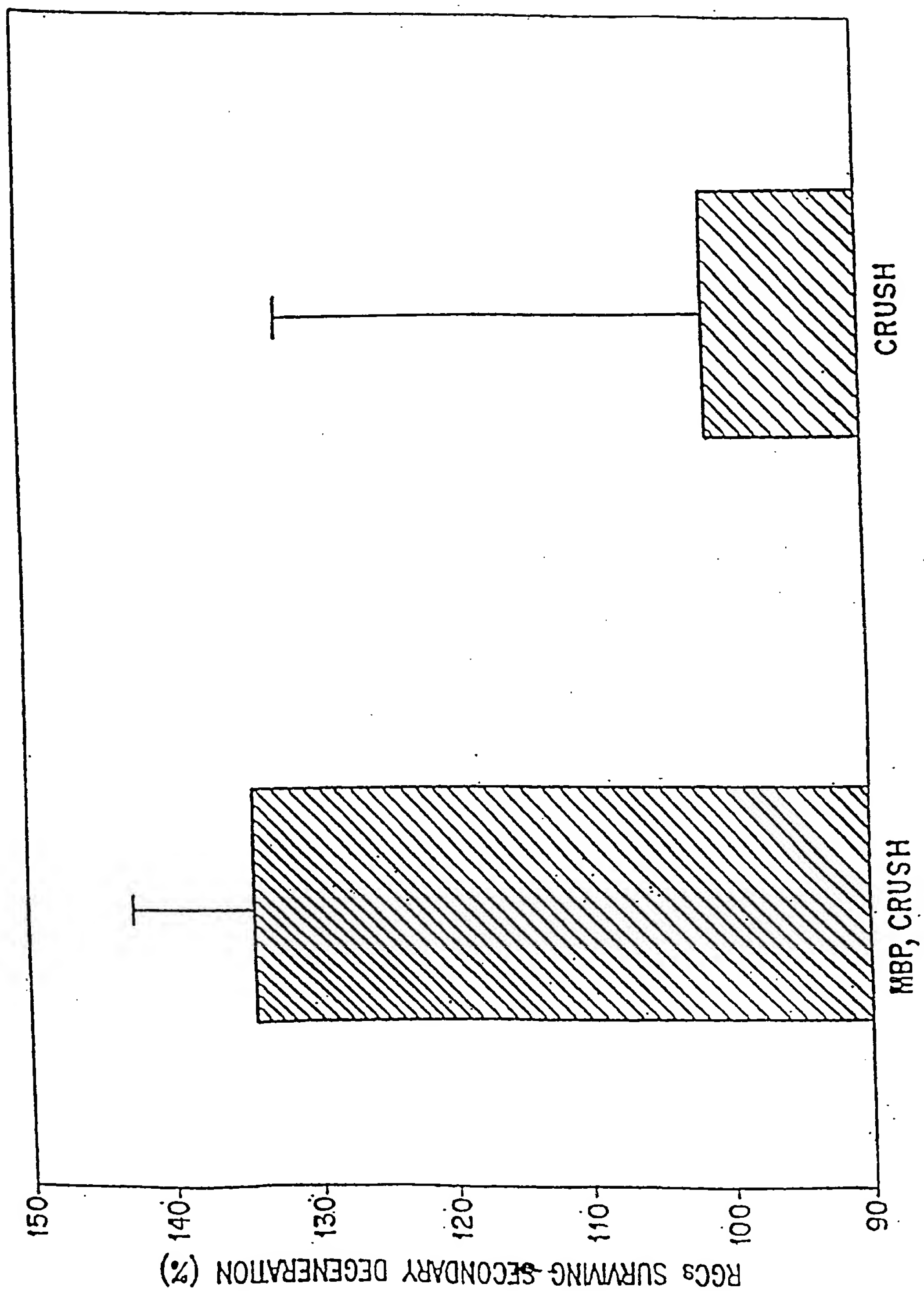

RGCs SURVIVING SECONDARY DEGENERATION (%)
150
140
130
120
110
100
90
MBP, CRUSH
CRUSH

FIG. 11

FIG. 12

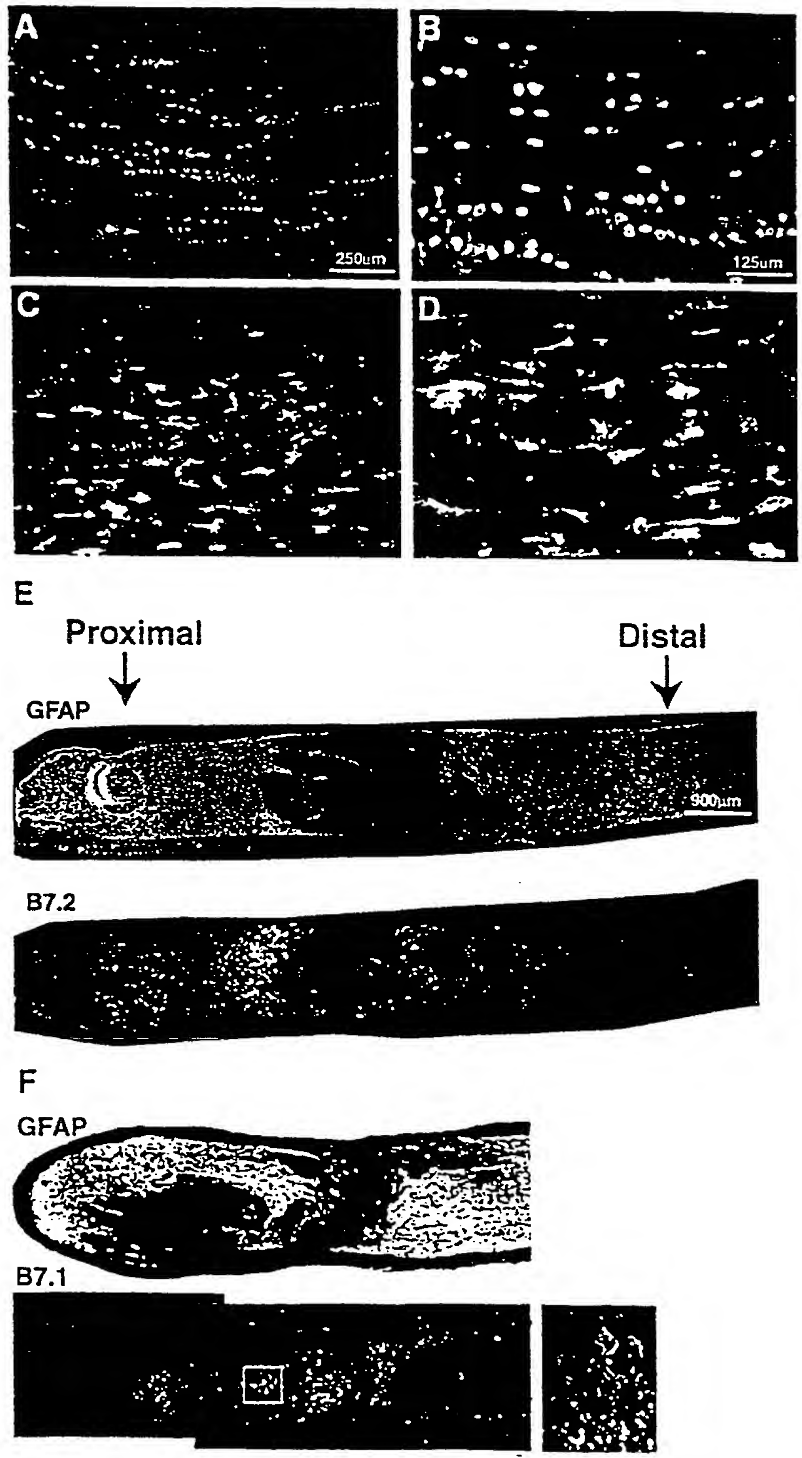


FIG. 13

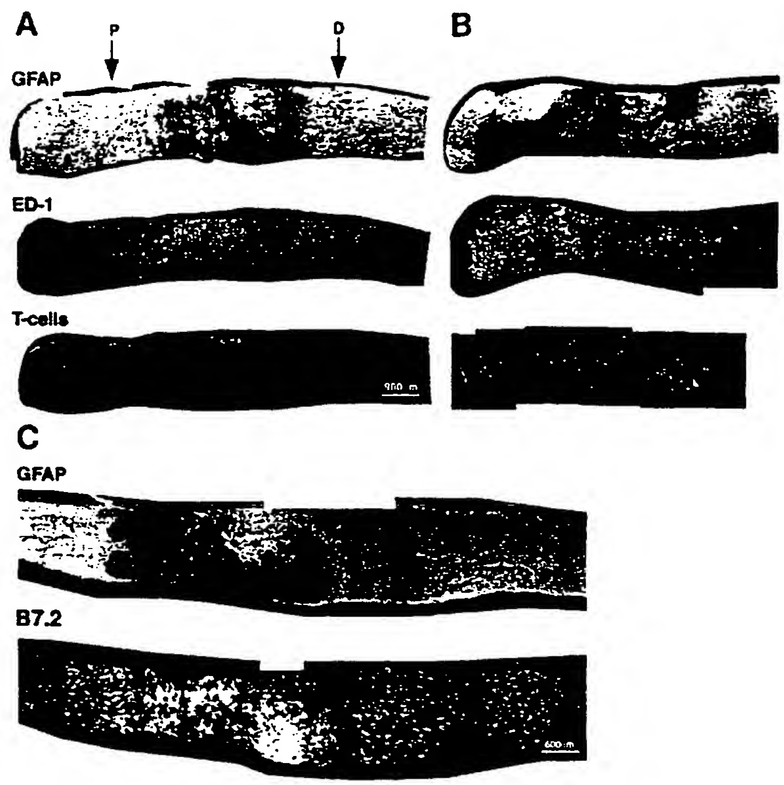
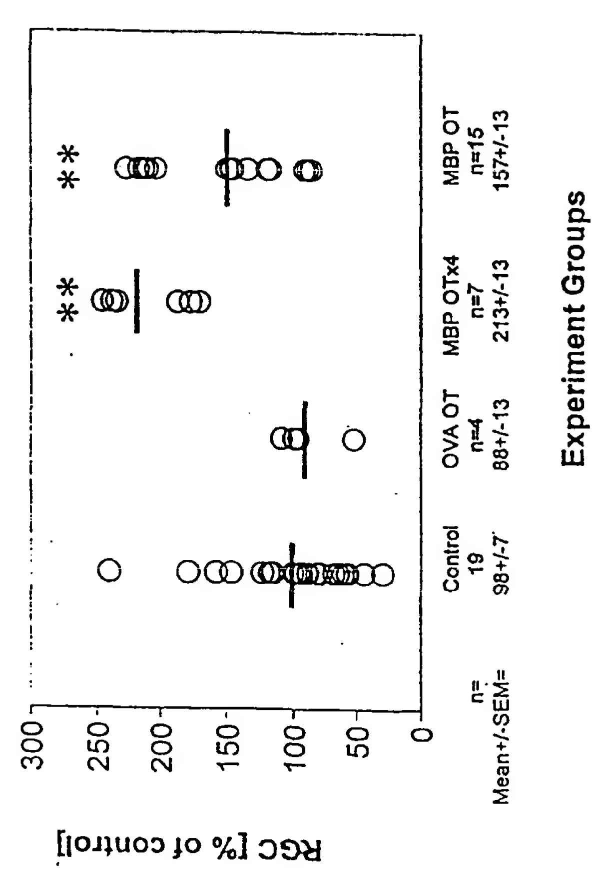


FIG. 14


RGC [% of control]
300
250
200
150
100
50
0
n=
Mean+/-SEM=
Control
19
98+/-7
OVA OT
n=4
88+/-13
MBP OTx4
n=7
213+/-13
MBP OT
n=15
157+/-13
Experiment Groups

```
  1 ccaagaagat cccacagcag cttccgaagg cctggatgtg atggcatcac agaagagacc
 61 ctcacagcga cacggatcca agtacttggc cacagcaagt accatggacc atgcccggca
121 tggcttcctc ccaaggcaca gagacacggg catccttgac tccatcgggc gcttctttag
181 cggtgacagg ggtgcgccca agcggggctc tggcaaggac tcacacacaa gaactaccca
241 ctacggctcc ctgccccaga agtcgcagag gacccaagat gaaaacccag tagtccactt
301 cttcaagaac attgtgacac ctcgtacacc ccctccatcc caaggaaagg ggagaggcct
361 gtccctcagc agatttagct ggggaggaag agacagccgc tctggatctc ccatggcaag
421 acgctgagag cctccctgct cagccttccc gaatcctgcc ctcggcttct taatataact
481 gccttaaacg tttaattcta cttgcaccaa atagctagtt agagcagacc ctctcttaat
541 cccgtggggc tgtgaacgcg gcgggccagc ccacggcacc ctgactggct aaaactgttt
601 gtccctttttt at
```

FIG. 15

```
   1 gaaaacagtg cagccacctc cgagagcctg gatgtgatgg cgtcacagaa gagaccctcc
  61 cagaggcacg gatccaagta cctggccaca gcaagtacca tggaccatgc caggcatggc
 121 ttcctcccaa ggcacagaga cacgggcatc cttgactcca tcgggcgctt ctttggcggt
 181 gacaggggtg cgccaaagcg gggctctggc aaggactcac accacccggc aagaactgct
 241 cactatggct ccctgcccca gaagtcacac ggccggaccc aagatgaaaa ccccgtagtc
 301 cacttcttca agaacattgt gacgcctcgc acaccacccc cgtcgcaggg aaaggggaga
 361 ggactgtccc tgagcagatt tagctggggg gccgaaggcc agagaccagg atttggctac
 421 ggaggcagag cgtccgacta taaatcggct cacaagggat tcaagggagt cgatgcccag
 481 ggcacgcttt ccaaaatttt taagctggga ggaagagata gtcgctctgg atcacccatg
 541 gctagacgct gaaaacccac ctggttccgg aatcctgtcc tcagcttctt aatataactg
 601 ccttaaaact ttaatcccac ttgcccctgt tacctaatta gagcagatga cccctcccct
 661 aatgcctgcg gagttgtgca cgtagtaggg tcaggccacg gcagcctacc ggcaatttcc
 721 ggccaacagt taaatgagaa catgaaaaca gaaaacggtt aaaactgtcc ctttctgtgt
 781 gaagatcacg ttccttcccc cgcaatgtgc ccccagacgc acgtgggtct tcaggggccc
 841 aggtgcacag acgtccctcc acgttcaccc ctccaccctt ggactttctt ttcgccgtgg
 901 ctcggcaccc ttgcgctttt gctggtcact gccatggagg cacacagctg cagagacaga
 961 gaggacgtgg gcggcagaga ggactgttga catccaagct tcctttgttt tttttcctg
1021 tccttctctc acctcctaaa gtagacttca tttttcctaa caggattaga cagtcaagga
1081 gtggcttact acatgtggga gctttttggt atgtgacatg cgggctgggc agctgttaga
1141 gtccaacgtg gggcagcaaa gagagggggc cacctcccca ggccgtggct gcccacacac
1201 cccaattagc tgaattcgcg tgtggcagag ggaggaaaag gaggcaaacg tgggctgggc
1261 aatggcctca cataggaaac agggtcttcc tggagatttg gtgatggaga tgtcaagcag
1321 gtggcctctg gacgtcaccg ttgccctgca tggtggcccc agagcagcct ctatgaacaa
1381 cctcgtttcc aaaccacagc ccacagccgg agagtccagg aagacttgcg cactcagagc
1441 agaagggtag gagtcctcta gacagcctcg cagccgcgcc agtcgcccat agacactggc
1501 tgtgaccggg cgtgctggca gcggcagtgc acagtggcca gcactaaccc tccctgagaa
1561 gataaccggc tcattcactt cctcccagaa gacgcgtggt agcgagtagg cacaggcgtg
1621 cacctgctcc cgaattactc accgagacac acgggctgag cagacggccc ctgtgatgga
1681 gacaaagagc tcttctgacc atatccttct taacacccgc tggcatctcc tttcgcgcct
1741 ccctccctaa cctactgacc cacctttga ttttagcgca cctgtgattg ataggccttc
1801 caaagagtcc cacgctggca tcaccctccc cgaggacgga gatgaggagt agtcagcgtg
1861 atgccaaaac gcgtcttctt aatccaattc taattctgaa tgtttcgtgt gggcttaata
1921 ccatgtctat taatatatag cctcgatgat gagagagtta caaagaacaa aactccagac
1981 acaaacctcc aaatttttca gcagaagcac tctgcgtcgc tgagctgagg tcggctctgc
2041 gatccatacg tggccgcacc cacacagcac gtgctgtgac gatggctgaa cggaaagtgt
2101 acactgttcc tgaatattga aataaaacaa taaactttt
```

FIG. 16

A.

```
   1 taatatctag ggktttgact ctgacccgtg ttggggctct cacttcatgg cttctcacgc
  61 ttgtgctgca tatcccacac caattagacc caaggatcag ttggaagttt ccaggacatc
 121 ttcattttat ttccaccctc aatccacatt tccagatgtc tctgcagcaa agcgaaattc
 181 caggcaagcc ttagggaaaa aaggaaaaac aaagaaaatg aaacaattgg cagtgaaagg
 241 cagaaagaga agatggagcc cttagagaag ggagtatccc tgagtaggtg gggaaaaggg
 301 gaggagaagg ggaggaggag aggaggagga aagcaggcct gtccctttaa gggggttggc
 361 tgtcaatcag aaagcccttt tcattgcagg agaagaggac aaagatactc agagagaaaa
 421 agtaaaagac cgaagaagga ggctggagag accaggatcc ttccagctga acaaagtcag
 481 ccacaaagca gactagccag ccggctacaa ttggagtcag agtcccaaag acatgggtaa
 541 gtttcaaaaa ctttagcatt gaagattcaa gaggacacag g
```

B.

```
   1 ctgctttcag agcctgtgac ttcttgtgtg cctctcctgt ttctcagcaa catggcatag
  61 ggcctgggat accaggtctg gggatctcag ggactcttag cactttaaga cacatgtgtt
 121 cccaggccct ggtgtgttcc tctagtgcca gaaagatgtt tcatgctttg ctgactttgt
 181 ataaagtctg tttgtagctg ttttgacaga atctcagcgt ataactgagg gtggggacat
 241 tagccaagct gcattatagg aggacaaaac tgccatacaa agtgtccaaa atcattaagc
 301 ctgcattttt attattggga gtaatatcaa acctcctatt ttccaatttt catttcttgt
 361 cctgtgctag ctccatcctg tttggactgc tcctcccata tgtaaactaa gaagaatcaa
 421 gcattctttg caacaaatac acacgatgct caaaaatgtc caggagcatc caatttccaa
 481 agtttcctcc acctggaatg ctcttcatgc taaaatcctg tctgacaata ccagcatctc
 541 tggcctgcac tcatcccttc ctggaactcc aagtgcattt accctctgtt accacttact
 601 tggctgcctg aattgttagt tgaaaatatt aggtctactt agctaattct tcctcaggaa
 661 attaaagact cccatatggc agagtctgtg tctttctct cttcatatcc cgtataacac
 721 ccagcataat gctgggcata tagtgagtat tccataaata gttgatgaat gactaaaata
 781 agcaagcaaa caaacagact agaacaataa gaaagaaggg actggatttc ataatctctc
 841 tggcttgcta tttgaattgc tgaattatta ttatttatta aatattttt aaattctggc
 901 aataaaaggt aaggatttat tttctttctt tctttttttt tttcttgaga cagagtctcg
 961 ctcttactgc ccaggctgga gtacaatggc gcaatcttgg ctcacggcaa cctccgcctc
1021 ctcctgggtt taacagattc tcctgtctca gcctcctgag tagctgggat tacaggcata
1081 cgcccatgcc cggctaattt ttgtattttt agtagagacg gggtttgcc atgttggcca
1141 ggctggtctt gaactcctga cctcatgtga tccacctgcc tcagcctccc aaagtgctgg
1201 gattacaggc atgcgccacc gtgcccggcc aaagatttat tttcaagaat gaaacaaagt
1261 aaggattctg ggtcaatctc acatgctgaa agccaaaacc tctagccgct cctgcttttt
1321 gacttcggag tgcccactat ctccgagcct gtgagcacag ggcctggcag aggggtttga
1381 gtggcatgag ctacctactg gatgtgcctg actgtttccc cttcttcttc cccaggcttg
1441 ttagagtgct gtgcaagatg tctggtaggg gcccccttg cttccctggt ggccactgga
1501 ttgtgtttct ttggggtggc actgttctgt ggctgtggac atgaagccct cactggcaca
1561 gaaaagctaa ttgagaccta tttctccaaa aactaccaag actatgagta tctcatcaat
1621 gtgtaagtac ctgccctccc acacagaccc atcttttttt tccctctctc catcctggag
1681 atagagaact cttcagtacc ttagtaacta gcaggggact ggggtggagc cagaccggat
1741 tcccgagtct tccctctgtg ca
```

FIGS. 17A-B

C.
```
  1 ctagaaaatc cctagccttg ttaaggtgct cgctctggtg tatacctcac ttatgtcggg
 61 aaagaagcca ggtcttcaat taataagatt ccctggtctc gtttgtctac ctgttaatgc
121 aggatccatg ccttccagta tgtcatctat ggaactgcct ctttcttctt cctttatggg
181 gccctcctgc tggctgaggg cttctacacc accggcgcag tcaggcagat ctttggcgac
241 tacaagacca ccatctgcgg caagggcctg agcgcaacgg taacaggggg ccagaagggg
301 aggggttcca gaggccaaca tcaagctcat tctttggagc gggtgtgtca ttgtttggga
361 aaatggctag gacatcccga caaggtgatc atcctcagga ttttgtggca ataacaaggg
421 gtgggggaaa attgggcgcg agtctgtggc ctcgtcccca cccaaggctg ggtcctctct
481 aggggcctgg catttgagtg aggaagcgat ggctgcagcc gaacgagaag gtcaggaaga
541 acgtggtgcc cagctggctt agcctcacct ttcaaaggtt ccctaagcaa atttcttctc
601 aaaacagaaa gcatgagttt tgtgggatgc tttgtacaat cagaccattt ctaagccatc
661 tgttggtatc cctttgttcc cttcctagta ggtaccacaa gagtggatct aactggacaa
721 gagtctaaaa tgctgctcat gtgattgaga cttgggcacc tgagctraga gggaggatgg
781 ataataaaaa ttaaataata actccaaggt aaatttacaa tgttctgg
```

D.
```
   1 gatcctcctc attcttcccc tacccattcc ccccaccctc cgttatactg ggccagtta
  61 tctagtagat actgccaatt acccttggca gaggtgccct gctcactaat tttatttggg
 121 ggagmgccct ggaacctggt tttaatgtct ggcacacgcc acttccagga tctcccagtt
 181 tgtgtttcta catctgcagg ctgatgctga tttctaacca acccatgtca atcattttag
 241 tttgtgggca tcacctatgc cctgaccgtt gtgtggctcc tggtgtttgc ctgctctgct
 301 gtgcctgtgt acatttactt caacacctgg accacctgcc agtctattgc cttccccagc
 361 aagacctctg ccagtatagg cagtctctgt gctgatgcca gaatgtatgg tgagttaggg
 421 tacgggtgct ttggctctcc tacccactat ggaagcacta tatatttggt tattttctta
 481 gtgtaaggag ggtggtgatt atgagaaaaa tataagatga tgaatgattg ggtcttagtt
 541 tattaatcct tccctactga aaccagagag gtttcttccc ccggaaggga acttggaagt
 601 ggtgggagtt ttcttggcca ttcacattgg cctactctag ttgactgctg ttcacaaccc
 661 caaagcagca catttcaata acaaacacaa ggttdsacca ctgttcaata ccaccttctc
 721 tttttgtaa acctgtagaa aagaggatcc taattgttgg tagmatccaa mtttacagcc
 781 aggataatta gagatggaag aagggctctg ggggaaagtc tccatgtggc cccgtaactc
 841 cataaagctt accctgcttg ctttttgtgt cttacttagg tgttctccca tggaatgctt
 901 tccctggcaa ggtttgtggc tccaaccttc tgtccatctg caaaacagct gaggtgagtg
 961 ggttatttgg gttattttac aagggagtag ctaataccat acaaattaca cccatggcct
1021 tcaattttaa ggactgaaag tttccctttg ctggattttg aattagccga ttgccttcta
1081 caacatgttg gctaagtgtg cctgagccaa tgagcataga aggtaaaaca cctcttttct
```

E.
```
  1 aattagcaca cagaaaggat atccaacaca tacaaagctg tnntcatgga ctacactgga
 61 gcatattact gctgttgcaa gaaacatttc ttcttcctct tttcattttc ctgcagttcc
121 aaatgacctt ccacctgttt attgctgcat ttgtgggggc tgcagctaca ctggtttccc
181 tggtgagttg actttgaatg atcttggcaa gtaaataggc ctgagatagt tgtgggtaca
241 gctattctga aaggcaagaa ggtagactgc ttccatcctt gaaatgctgg aggga
```

FIGS. 17C-E

F.

```
   1 aattctatat actatcacta tggctccact ttggatactc tccagtggat ttagttactc
  61 atatggaaat acctgggagg acctcctaac attattagaa ttgttatgat tataatacaa
 121 ygctatgtcc caggtcttgc tgatagtgct acagtgccct gtgaatgtag tgtgctcatt
 181 gtgcagatta aaaacctaag gcactgaagg gtgaagtgat ttatctgaag ttattttata
 241 aagcagtgat cagacaasct gagctcacag aactccctgg cccctactgc tgaggtttcc
 301 atacagagtc aagtaatttc tcaccttgta aaacgaattg attcattaac caggggagag
 361 ctctactgca tgatgtggct gtgtgtctac agcaagcacc ctatgactct aagtcactcg
 421 gacatattga tgtggcaaag cccaaatatt gttcacttcc ctgaggaaaa ctcagtgcta
 481 gatcaaacag aggtgtggaa taaatcttta tgatttgatt ctctgggcct gggccatgag
 541 acccatgatg cctcagagac atcggacttc cagtcaagtg tatatggaga aagccaagcc
 601 tgggatgtac tgctttttgc agagcatggg tttttccctt atttagttat gattttattt
 661 ctacccttcc tcattcccaa agggatttga ggagggagtg ctttcttttc tactctcatt
 721 cacattctct cttctgttcc ctacagctca ccttcatgat tgctgccact tacaactttg
 781 ccgtccttaa actcatgggc cgaggcacca agttctgatc ccccgtagaa atcccccttt
 841 ctctaatagc gaggctctaa ccacacagcc tacaatgctg cgtctcccat cttaactctt
 901 tgcctttgcc accaactggc cctcttctta cttgatgagt gtaacaagaa aggagagtct
 961 tgcagtgatt aaggtctctc tttggactct cccctcttat gtacctcttt tagtcatttt
1021 gcttcatagc tggttcctgc tagaaatggg aaatgcctaa taatatgact tcccaactgc
1081 aagtcacaaa ggaatggagg ctctaattga attttcaagc atctcctgag gatcagaaag
1141 taatttcttc tcaaagggta cttccactga tggaaacaaa gtggaaggaa agatgctcag
1201 gtacagagaa ggaatgtctt tggtcctctt gccatctata ggggccaaat atattctctt
1261 tggtgtacaa aatggaattc attctgcgtc tctctattac actgaagata gaagaaaaaa
1321 gaatgtcaga aaaacaataa gagcgtttgc ccaaatctgc ctattgcagc tgggagaagg
1381 gggtcaaagc aaggatcttt cacccacaga aagagagcac tgaccccgat ggcgatggac
1441 tactgaagcc ctaactcagc caaccttact tacagcataa gggagcgtag aatctgtgta
1501 gacgaagggg gcatctggcc ttacacctcg ttagggaaga gaaacagggt cttgtcagca
1561 tcttctcact cccttctcct tgataacagc taccatgaca accctgtggt ttccaaggag
1621 ctgagaatag aaggaaacta gcttacatga gaacagactg gcctgaggag cagcagttgc
1681 tggtggctaa tggtgtaacc tgagatggcc ctctggtaga cacaggatag ataactcttt
1741 ggatagcatg tctttttttc tgttaattag ttgtgtactc tggcctctgt catatcttca
1801 caatggtgct catttcatgg ggtattatcc attcagtcat cgtaggtgat ttgaaggtct
1861 tgatttgttt tagaatgatg cacatttcat gtattccagt ttgtttatta cttatttggg
1921 gttgcatcag aaatgtctgg agaataattc tttgattatg actgtttttt aaactaggaa
1981 aattggacat taagcatcac aaatgatatt aaaaattggc tagttgaatc tattgggatt
2041 ttctacaagt attctgcctt tgcagaaaca gatttggtga atttgaatct caatttgagt
2101 aatctgatcg ttctttctag ctaatggaaa atgattttac ttagcaatgt tatcttggtg
2161 tgttaagagt taggtttaac ataaaggtta ttttctcctg atatagatca cataacagaa
2221 tgcaccagtc atcagctatt cagttggtaa gcttccagtc atcagctatt cagttggtaa
2281 gcttcccagg aaaaaggaca ggcagaaaga gtttgagacc tgaatagctc ccagatttca
2341 gtcttttaat gtttttgtta actttgggtt aaaaaaaaaa aaagtctgat tggttttaat
2401 tgaaggaaag atttgtacta cagttctttt gttgtaaaga gttgtgttgt tcttttcccc
2461 caaagtggtt tcagcaatat ttaaggagat gtaagagctt tacaaaaaga cacttgatac
2521 ttgttttcaa accagtatac aagataagct tccaggctgc atagaaggag gagagggaaa
2581 atgttttgta agaaaccaat caagataaag gacagtgaag taatccgtac cttgtgtttt
2641 gttttgattt aataacataa caaataacca acccttccct gaaaacctca catgcataca
2701 tacacatata tacacacaca aagagagtta atcaactgaa agtgttccct catttctgat
2761 atagaattgc aattttaaca cacataaagg ataaactttt agaaacttat cttacaaagt
2821 gtattttata aaattaaaga aaataaaatt aagaatgttc tcaatcaaac atcgtgtcct
2881 ttgagtgaat tgttctattt gacttcacaa tagaaactta ataatcgtac cttctcaaga
```

FIG. 17F

1 atggaaatgt tctgtatttg tgttgtctga tgagataacc actaactgta gtgctattga
61 gcatttgaaa catggctagt gtaatcaatg aaccaaattt ttaattttat ttaattgtaa
121 ttaattttaa gtggccacat gcagggagtg actgctgcat tggacagcac ggctctaaat
181 tgagcctttt ttccttattt ggtgaggcat acttgcctta agattgggaa gtctattttt
241 ggaacctgct accaatgctg gtctcacact tgcaattctc agctgagcca agaggtgaga
301 gaaaggtcat tttccattcc aagatctcac tctcccctgt gacactgagg aaactggcaa
361 gtgatgtgaa ggctggagag cgtgtcctgt atgctggctc tgtcccttct gcctgtgttg
421 actgacatag ttagttgctg cccttgctgg tctcccttcc tccaaccttg cctctctgag
481 cacacctgac attcatctca tgacttccct aaaaacattc tttgggaaca agaaactaac
541 aaatcccaag tgacctatca catatacaaa catacagggc agagtttgga ttcgcggtag
601 aagaaaggga ggttagacat taagaagaat ggtctggtga tgacagttgt gagataatag
661 aaacaggaaa aagaaatcta agtttctttt cttttttaa gaaccaataa taatttctct
721 cttttgacta gtcagtaggg ctggggtgga ttggaggaag cttacatatt ccatgaacaa
781 gcctcttcct aaggtcctgt aagtgatcct gccccactga ttagccccta gaagaccctt
841 caaaggttgg atctccagga gggagtgggg gaggaaagcc ctgtaccagg cagcctctgc
901 tccattgctc tgggggggtg gggaagacaa accctggtca tcccctcagt ctgtagccct
961 tttgtgtgag tgcctggcaa gggtgacgtg gggctgtttc tgcgggcaca gctgcagcaa
1021 ttaccggagt ggaggcaggg cccaggcagc actgccctcc aagatcttcc cttgggcttt
1081 tcagcagtaa ggggacatgc accccaaggg cctccacttg gcctgacctt gctgcggggg
1141 ctctctgtcc ccaggaacag tagagatggc aagcttatcg agaccctctc tgcccagctg
1201 cctctgctcc ttcctcctcc tcctcctcct ccaagtgtct tccagctatg caggtaagac
1261 atgtttttt tcctgccctg gggagaccct gaaaacagaa aggctagttt cctgggggtt
1321 agctccttca aacatcctca agttggtata ttatctttct aaaacataga cctactgaca
1381 tgcctccctt cctcagaaac cttccgtggg tggttcttac agccttcaag atggagtcca
1441 gactcttttt ttttttggg acagagtctc cctctgttgc tcaggctgga gtgcagtggc
1501 atgatctcgg ctcactgcaa cctcagcctc cctggttcaa gcgattctcc tgacttggcc
1561 tcccaagtag cggagactac aggcgcctgc caccacaccc agctaaattt gttcttttct
1621 ttcttttttt tttttttgg gattttagga cagacggggt ttcacatgtt ggccaggatg
1681 gtctcgatct cttgacctgc tgatccgccc gcctcagctt cccaaagtac tgggattatg
1741 ggcgtgagcc actgcactag gcctaatttt tttattttta gtagagatgg ggtttcacca
1801 tgttggccag gctggtctgg aacccctgac ctcaagtggt ctgccctcct cagcctccca
1861 aagttctgag attacaggca tgagccattg cgtctgaccc agactcctta atgtgactaa
1921 ctccaggctt tccttggact acttcttact tgtctttcca gctttgtctt ttcacctctc
1981 caattgagat aaaataataa caacctcttg gagttctcat caggattaca tgaaatgaga
2041 tatgtaacat gcttagcagt gcctgtccat agtaaatctc aataaatgtt tgtggaatta
2101 taatatcttg tcatgtttga gactttgctc tgcataatca ggcaccagta ggtttttata
2161 aaggaacccg tctgtcacgt gcagaggaga aataaacaga aagtttccca tcctcaggga
2221 gccacctgac tgacagaggc acagtgcatc cactctccag gtctagggga gaaagcagcc
2281 ttatttctta gtagctcaga atctgacttg agaaacacat ccacatagaa aaaaacaagg
2341 aactttttcg ggtcagggtc cgggacccac agtgaggtgg aagatacagg ggaaggaaga
2401 gggaaataga gccatcccca gggtggaaga tctcagaaga gaatttggga aacaaggtat
2461 gaacaaggac tgaatagtga gaagtgatgg agagacagct aaagtagatg gagtgtcaaa
2521 accaaaacct ctaagggtag aataggcagc aatttggcca agtcctaaca gggaggccca
2581 taggaggatt caacctcaag atgctgtgcc acattccaag agggaaccta aaggctgggc
2641 tgaagagtca gagatggcta cagctggcaa aaagatgggc agatgctgag aggagatgat
2701 tgctaaaatg ttctgtccag gacattcaca gtatctctat aaccagagtc tttttgtcg
2761 ttgttgttct caagaaggaa acttgaggcc gggtgtggtg gtttatgccc ataatcccag
2821 cgctttgggg ccaaggcagg cggatcacct gaggtcagga gttcgagacc agcctggcca
2881 acagtgtgaa acctcatctt tactaaaaat acaaaaatta gctggatgcg gcggtaggtg
2941 cctgtaatgc cagctactcg ggaggctgag gcaggagaat cacttgaacc tgggaggcgg
3001 aggttgcagg gaggcggagg ttgcagtgag ccaagattgc accactgcac tccagcctgg

FIG. 18

3061 gcgacagaga gtaagactgt ctcaaaaat aaatgaataa ataaaagga agaagaagaa
3121 gaagaacaat tgcaatcctc cctggctcta gaatgtcatt taaaagtcga gtgtcttctt
3181 ccttccctgt tttgaagcag cccttctcat gacaggcttg cttgccaagg ttccctctga
3241 ccttaaatct cttcctttg gtgtcttgga cagggcagtt cagagtgata ggaccaagac
3301 accctatccg ggctctggtc ggggatgaag tggaattgcc atgtcgcata tctcctggga
3361 agaacgctac aggcatggag gtggggtggt accgcccccc cttctctagg gtggttcatc
3421 tctacagaaa tggcaaggac caagatggag accaggcacc tgaatatcgg ggccggacag
3481 agctgctgaa agatgctatt ggtgagggaa aggtgactct caggatccgg aatgtaaggt
3541 tctcagatga aggaggtttc acctgcttct tccgagatca ttcttaccaa gaggaggcag
3601 caatggaatt gaaagtagaa ggtgagtagt gccatataat attaggtatt aactgttggg
3661 tggccaagaa caattattct ctcaactgag atgagatccc tcaacccaaa catctcagtc
3721 ctgggaatga tttccataaa aatgtacaca tcaataaaca gaaactcatg cttagggatg
3781 tctgttgcat cattattcag agtagcaagg aaattgggat caaatcaat gcctttgagt
3841 aggtaagtga cagaatgaac aatggtagcc atactgtgaa tattatgcag ggattaaaaa
3901 gattatttta gcactaggcc agatggtttg gggggctcct ctaaggtatt attgagtgat
3961 aagagcaagc tgctgtagga tacaaaaaca aaaacaaaac cctagggcat ggtggtttgc
4021 ctcgcagcta ctcaggaggc tgagacggga ggctggcttg agcccagggg tttgcagtta
4081 cagtgagcta tgattgcacc actgcactcc aacccgggtg acagagcaaa gaccttcacc
4141 cccactccct acccgtctct aaaaaaaaca aaaacaaaaa caaaaaaacc cttgggccca
4201 gcgccgtggc tcacgcctgt aatcccagca ctgtgggagg ccgaggtggg cagatcacaa
4261 ggtcaggaga tcgagaccat cctggctaaa acggtgaaac cccgtctcta ctaaaaatac
4321 aaaaaaaaaa aaaaaattta gccaggcatg gtagcaggcg cctgtagtcc cagctactcg
4381 ggaggctgag gcaggagaat ggcgtgaacc cggaagcgga ggttgcagtg agccaaaatc
4441 cttccactgc actccagcat gggggacaca gcgagactcc gtctcaaaaa aaaaaaaaaa
4501 accctgtatt tgtgagcgca cacacacaca cacacacaca cacacctgtg cttggtccta
4561 gtgaataagc aagtaaatca aatgtctaaa tataattata gaaaggagat gtcacctttt
4621 ggctgtacct ccactatttc attctgcaga attgcagaat ttcttttttt tttcctttct
4661 ttcttttctt tttttttttg acacagagtc tcgctctgta acccaggctg gagtgcaatg
4741 gcgccctccg cctcctgggt tcaagtgatt ctcctgcctc agcctcccga gtagctggga
4801 ttacaggtgc ccaccaccac acccagctaa tttttgtatt tttagtagag acagggtttc
4861 accaggttgt caaggttggt ctcaaactcc tgacctcagg tgatccactc gcctcagact
4921 cccaaagtgc tgggattaca ggcatgagcc atggtgcccg gcctcagaat ttcattttca
4981 acatgttttg catgatgggt gattttggag aatatttttt gctctatcgc aggatgatta
5041 agatgtggac aaggtgaagc cgatggaggg ggagctttga aagttacttg ctatttaatt
5101 gaggaactaa actgctttga gagcctgggg gtcagatcct ctgccttttc ctcctcccca
5161 cctgcagtgc aaacatcaga caattgatca ctattgtatc ttggaggtgg gagtgaccat
5221 tgcagtgctg ggaccagaag atggcattgt atgtggaaca acaaagcact atttctagag
5281 actgcctgca gggatatgga aatagcttta tgtgtctcag aatgttcttc atacagctgt
5341 ttttattggg gaaattctac ttgccgaaaa gtttgatagt gagaccctct ccagtttgca
5401 gatttttctc cttcctgctc aacaacttcc tagctcagta actgcctctc ccaacaaact
5461 ccctcagttt caccacacca aaaaaggaag acaagccggt tgcggtggct cacacctata
5521 atcccaaaac tttgggaggc cgaggcgggt ggatccacct gaggtcggga gttcgagact
5581 agcctgacca acatggagaa accctgtctc tactaaaaac acaaaattag cctggcgtgg
5641 tggcgcattc ctgtaatccc agctgggagg ctgaggcagg agaatcgctt gaaccccgga
5701 ggcggaggtt gcagtgagcc aagatcgttc cattacactc cagtctgggc aagaaaagtg
5761 gaactccatc tccaaaaaaa aaaaaaaaaa aacaaggaag acaaaagaa aagcagctaa
5821 agactttgcc tcaggggaga aagttctctt ttgggttgct atccacattc caacctcctg
5881 ttcccacctc ttcgtctgca tgcctaagaa actgttttac aagtaaataa gggacgcttt
5941 gtctaggctt tggagccagg aagttgagac aaatttagga atgagatgaa gtaatggtat
6001 tattgcaagt ctcaggtgta actacctctg ctctttctct gaagagtttc taatttctct
6061 tgtttactta ttttttctt gtcattttg ggattttatt actagttgtc tctaatcctt

FIG. 18 (cont.)

6121 tctttaaatt cttcattatg aaacataaaa acaaatgcca ggcgcggcag ctcacgcctg
6181 taatcccagc actttgggag gccgaagcgg gcagatcacc cggtcagga gttcgagacc
6241 agcctgatca acatggagaa accccgtctc tactaaaaaa tacaaatta gctaggcgtg
6301 gtggcacatg ccagtaatcc cagctacttg agagactgag gcaggagaat cgcttgaacc
6361 gggaggcaga ggttgcggtg agccaagatc gcgccattgc actccagcct gggcaacaag
6421 agcaaaactc tgtctcaaaa aaaaaaaacc acatacaaac cagagataat attataatga
6481 gcctccaagt gcctaccacc ttgctgcagc acttgtcaat ccagggacca cccacctcac
6541 cggctcccca ctcattacca ccctccccta ctcaattact gaggtaaatc ctaggcagca
6601 tgatcatttc tttttttct ttttatttat tttgagacag gatctgtctc tgtcacccag
6661 gctggagtgt agtggcatat ctctgctcac tgcagcctct gcctcccggg cagaagccat
6721 cctcccacct cagcctacat agtagctggg accacaggca cacaccacca cacactgcta
6781 atgttttgta tttttgtag agactgggtt ttaccatgtt gatcaggctg gtctcaaact
6841 cctaggctca agcaatcctc ccacctcggc ctcccaaagt gctagaatta caggcgcgag
6901 ccactgcacc cagcgaagaa cactttttaa aaaataaata ggccgggcgc ggtggctcac
6961 acctgtaatc ccagtacttt gggagcccaa ggagggcgaa tcatgaggtc aagagattga
7021 gaccatccta agtaacatgg tgaaacccca tttctactac aaatacaaaa acaaaattag
7081 cctggcgtgg tggcaggcgc ctgtagtccc agctacttgg gagctgaggc aggagaatgg
7141 agtgaacccg ggaggcggag cttgcagtga gctgagatca tgccactgca ctcccccctg
7201 gggcaacaga gtgagactcc caaaaaaaaa aaaaaaagcc cccccctcccc acacacaata
7261 atataaataa ataaataacc acaatactat tatcacatct tacaaactca acaaaaattt
7321 cttaatatca tcaaataccc agtttgtgtt caaattttcc tgattgtttc ataaatatac
7381 tcttacagtt ggtttctttt agcgagattc aaatgagacc cacctgttga cctttgccct
7441 tagggtttcc cagggtctga attttgttga cgacattccc atgttgctat gtaatacggt
7501 cctccatgcc ctgtgttttt ctgtaaactg atagatgtgg aggtgcaatc acatttgtgt
7561 ttgatttact ttggcaaata tagttcatca gtgatactct atacttcttg ttgctttaca
7621 tccggaggct gataatgtct gcttttctct cttttctaat tatttgtgaa aggaaaaatg
7681 tgggggttg ggagaaaaaa acccttaagt acatactcgc taaatcacat tgctacaggt
7741 aacttccatt aagaacttga aagtaaaggt agctgcattt tcccctaggg aacacaatga
7801 tagacaggag ccttagtcta cagcttgaag gattgtaatt atacctaagc aaccctcctg
7861 gaccagttta atgttattag ctgtgatgta tccctacctt tgatgtcatt atccttactt
7921 agctccctta aagcagagat caagatgaaa agggcttcag ctgcagcatg gcacatggag
7981 attagagtgg ggcttttgga tgctgaggag cagacctaga atgggaaata gatgggagcc
8041 acagaagtga aggtccccct ccctcattgc tcaacctact ccacatctcc aggtctgcac
8101 atctgttcag ttactgaatc ctgtgtaagc taccttcttt ttcttttttc ttttatttat
8161 ttatttattt ttttttgag atggagtttt gctcttgtta cccaggctgg agtgcaatgg
8221 tgcaatctcg gctcactgca ccctccaact cccaggttca tgcaattctc ctccctcagc
8281 cttccaagta gctgggatta caggctgcac caccatgtct ggctaatttt tgaaaaatca
8341 gtagagagag ggtttcacca tgttggccaa gccggtctcg aactcctgac ctcaagtgat
8401 ccacccacct tggcctccca aaatgctggg attacaggtg tgagccacca tgcccgctgt
8461 aaactacctt cttaaaagct ctagaagagg gcttttaacc ttttgttgtg tgtcatgcac
8521 cttccgcaag ctgatgaagt tgatagaccc atctcagaat tttttttttt tttttgagac
8581 agtgtctcac tctgtcaccc aggattggtt gcagtggcac gatcatgggt cattgcagcc
8641 tccacctccc aggctcaagt gatcctcctg actcagcctc ttgaatagct gagaccacag
8701 gcttgtgtca ccatgcccag gtaattttta attttttttc gtagaggcag ggtctcacat
8761 tatgttgccc agtctggcct cgagaactcc tgggctcaag caatcttcct gccttgggct
8821 cccaaagtgg tgggattaca ggggagagcc accacaccta gccaggagga tgttttaaat
8881 acaccaaata aaacatttat acccaaatac agttatccaa atattaaatt aacaagagtt
8941 agggtgaccc tattaattag tgtaatttcc aaatagtaat gaacataagt gatagtttga
9001 gatttctgtg acttttctaa tgtgacgtga aaatatttgt gatttttctt tttctttttt
9061 tttttgaga tggagtttcg ctcttgttgc ccaggctgga gtgcaatggc aagatctcgg
9121 ctcacctcaa cctccgcctc ctgggttcaa gcgattctcc tgcctcagcc tcttgagtag

FIG. 18 (cont.)

9181 ctgggattac aggactgtgc caccacgtcc agctaatttt gtatttttag tagaaacagg
9241 gtttctccat gttggtcagg ctggtcttga actcccaacc tcaggcgatc cgcccgcctc
9301 ggcctcccaa agtgctggga ttacaggtgt gagccaccgc acctggccaa tatttgtgat
9361 ttttattgac gacaaagtca aaggttctct tcatattatt gtggtgtatc gcctacaagc
9421 ataattaaaa taaacactaa atttcagttt aaagtttact gaaaataaat atgtattttt
9481 tattccctat ttaagctttg aatcccctga cttcctatac cattaccact gtcctagttc
9541 aggttcatgt tgttttttac tttaattgtt atcacagtct cttaacattt ctccctatgt
9601 tctccagtcc tgtaggtgct aaatctgacg tggtcacttc tcagcttgga atccttcagt
9661 gcaccaccac agccttgaac tacatatttg aaatacatat ttattttcag taaactttaa
9721 actgaaattt agtgtttatt ttaattatgc ttgtaggcga tacaccacaa taatatgaag
9781 agaacctttg actttgtcgt caataaaaag tcccttgagg ggacttcaga tgtaagtccc
9841 ttagctgctc gttaaaactc ccccaggctg acccaataca caatcttgac tttaaaccac
9901 ttgtcattct aaatcactag catttcctgg aaaaaaaagc catttttcct tcagggctaa
9961 gctcagggac caattctgtg tcaccttctt tgaatcctga tgatattcac ttctttattt
10021 gacctgattt attgggcccc agacaccatg ctgagtgttg gggattcagc tctggacaat
10081 gtcaaatgtc agtcctgcct ttcagatcct ttctactggg tgagccctgg agtgctggtt
10141 ctcctcgcgg tgctgcctgt gctcctcctg cagatcactc ttggcctcgt cttcctctgc
10201 ctgcagtaca gactgagagg tacagggcag agggtgggtg gatcaggatc ctttctttaa
10261 atgagctggc ttcttggagc tacaccactt aacatgtatt tgtgagtgac ttctgggttc
10321 agaagttctt ctcactattg agtgataaag aaaaaaaata actccatgat gaaagagttt
10381 tacatcttac ggaatgcttt catatgaata atcggaccta gcatttccct atgagctaac
10441 tatgccatat agtaacccca ttttacagag gatacaactg aggccaggag tagttcagtg
10501 acttactcaa accgatataa cttataagtg gtagagctga ggcctctgta tcatacctag
10561 cagctccatg caacttggga gagtgtgagc ttcgaagtca gacaggtcta ggctattagg
10621 agttttgaat aaagatactg aagtgaaagt ctctaccaca cagtaggcgt tcgaaaattg
10681 tttcctcttt ctccattcaa cactgaggac tcaggttcag ctgctgatga agctcctctt
10741 ttttgcctag agctttcatt ctgagccttc tcctcctacc aagtgtctcc ccaatgccag
10801 agcaggaaga gtcttcactc ctcccaatgc cccacctccc atttgttact aagaggagag
10861 gagaaagtag caaggagggt atggggaatg ttctgggggа atgggtgttg gtgcgatcaa
10921 caacaaagtc ctttctctca ccttgaattc atcccagatg cctgcttgtt tacttcttcc
10981 acacaaaaaa aggccttcag ccctcatggc tgagcagaaa gaatctgaat gttagagtca
11041 ggcagcctgg gtttgaattc catctcaggt actgaactct atagcaaaat tcttagattc
11101 tccaagcttc agttgccttg tctgtcaaat agagaaaaca tccttcgtcc taaattgtag
11161 ggaggattaa agtcatgcaa agtgcctact acaaatccag tcacaaagta gctagctact
11221 cactaaatgt tcagctcctc cctcctcatt cagatgggaa gtggctttag ataaacaaag
11281 tggcaacgca gtgggctgga gcagctctgt gaactgagaa tccaagaaaa ggggcgaaga
11341 gcagctggga tgtattggat gcttgtgctg gcttggagca ttgctcacat tctttattcg
11401 ctattgtatc tagactatag ctagagaaag agccgcaacc attggcttta aatccagtgc
11461 tcttcctact ctcctgaggt tgtttccagg ctgcagagaa atagcctgca caaggggccc
11521 aggcgctggg tgtgggaggg tccccaccga gagccagaac atgcaggaac taaaatgttg
11581 cctttttcta ttttaggaaa acttcgagca gagataggtg agttccagtc atcgtttctc
11641 ccaattcttg ccttttggtt ttttggcata acggaaatgg tcccattctt ggaccgtctc
11701 tccctctcaa taccctgttt tcccctcagt ttccctttct ctacagtggg tgtgtcgtgc
11761 ctagaacaag ttttaagtaa ttaaataaca aagactcagg ataaaaggat ccttttttgga
11821 gtgccctact aaatccattt ccatttgttt ctctttcaga gaatctccac cggacttttg
11881 gtaagttccg gcatgtctag gccctcccag gtcaacttgg tatttcactc tagttccagt
11941 cacctggggg aacaaggacc cctggctcct ggttgagtcc cttcctctct tctcttttct
12001 ttctttaaat aagaagtcat ttgcatttag gattggtaaa atcataataa aaatactcat
12061 gtactgtttt tatgtgccag gcactattct aactacttta caaaaacgtt atcttattct
12121 gtttaactcc ttatgcacat gatctctctt ttcaggaatg ccaaaacaca ggtaaataga
12181 tcgtttacac gtaaacctga tgtctggttg gggaggtgaa acaaacagaa acaagacaca

FIG. 18 (cont.)

12241 actgtatcac ctgtacttat atttctgctt tacaaactca ggatgtttcc atgagtacag
12301 aacatgacta atcagagaag acctcataga ggaatagaaa agccaccaag ccccactagg
12361 aattgacccc tcaaggacat ggtttctagc ctttttgttc actgcagatt gcccaatgcc
12421 taaagataat ggcaacagaa gagcacccaa atatttgtta gataaatgtt gcagacacta
12481 gaaggtgtca ttagggcaca gatggtacct tctctgagca aacttccttc acagctcctc
12541 ctcccgaggc tgtaggtgac tctactcttg tcacctggca cacagagttc tatcgtacga
12601 tttaggaaat tagaccagtg tgtggaccac acacacacac atctttacac acccaaagag
12661 gaggaatagt atctttgttt tggaggactt gactatgaaa ggtcttaact ccttttgta
12721 ccatgaatct ctctggcact ccagtgaagt ctaaaggacc cctttgcaga atgttttaa
12781 atatacacat aaaatagaac acataggatt gcaaaaacaa tcattgtact aaaatacagt
12841 tatcaaccga taatcacatt tgtgatatag taacataaat gtttcttttt tttttttttg
12901 gaggcagagt ttggctcttg tcacccaggc tggagtgcaa tggcgcgatc taggctcact
12961 gaaacctctg cctcccgggt tcaagcgatt ctcagcctcc tgagtagctg ggattacagg
13021 tgcccgccac cacacccagc taatttttgt atttttagta gagactaggt ttcaccaggt
13081 tggccaggct ggcctcgaac tcctgacctc aggtgatcca cctgccttgg cctcccaaag
13141 tgctgggatt acgggcatga gccaccgtgc ccggccataa atatttcttt agccaaagta
13201 atacattaag taatgtagca gcaagtctaa taacctgtaa tttctttctt tctttctttc
13261 tttctttttt tttgagatga agtttttttg agatggagtg caatggcaca atctcggctc
13321 actgcaacct ccacctcctg ggttcaagcg attctcctgc ctcagcctcc caagttgctg
13381 gaactacagg cgcatgccac catgcccagc taatttttgt atttttagta gagacggggt
13441 ttcaccatgt tggccaggct ggtcttgaac ccctgacctc aggtgatctg cctgccttgg
13501 ccttccaaag tgctgggatt acaggcatga gccaccaggc ccagcccaat aacctttaat
13561 ttcaacatac taataaacat aaacagtatt tcaagatttc tgcaataact ctaatgggaa
13621 tgaaaacatc tgtggcttcc attggtaatt aagtcacagg tactgctcat attgtggtta
13681 gttgtaaaat gttttggttt gttttgtttt ttccaagact tggggaatg ggtgttggtg
13741 ggatcaacaa gagtcttgct ctgtggccca ggctggagtg caggggcagg atcttggctc
13801 actgcaacct ccgcctccca ggttcaagcg attctcctgc ctcagcctcc tgagtagctg
13861 gcattacagg catgtgccac cacgcccagc taatttttac atttttagta gagatggggt
13921 ttcaccatgt tggcctggct ggtcttgaac tcttggcctc atgatccacc cgtctcggac
13981 tcccagagtg ttgggattac aggcatgagc caccacacct ggcagttgtt acattttttaa
14041 tgaaagaaaa tgttaaatcc agttattgaa aataaggagg cagtactttt ctcatccaag
14101 ttcatggact ttctgaattt tgtccccaga gtcctttggt gttctaggac cccaggttaa
14161 ggaacccaaa aagacaggtg ggtggggcat gagggggaac acatgttaat ccctgtttgt
14221 tctggtgaac aattcagatc cccactttct gagggtgccc tgctggaaga taaccctgtt
14281 tgtaattgtg ccggttcttg gacccttggt tgccttgatc atctgctaca actggctaca
14341 tcgaagacta gcaggtgcag tggctgggca gcaggcaaga ccaccaaata gtgggggacc
14401 aagtcagctc tgaatgggaa gccaaaagag aatagaacca ggactcaaga ttagggggagc
14461 tgggatttcc ttattcctct gtccccatgc ccaaccccag gctcttctga gaaactgtga
14521 agagaaccac ttactggatc tgtgggatcc cccagtggaa agggcagtgt gggtcactcc
14581 aaatgtccat agggaggatg tggggaaggt gctattcatc ttccactaat cacatatttg
14641 tttctttttg ttttcagggc aattccttga agagctacgt aagttctctt ctctctgtta
14701 taagcagaga ataaaaagcc aggaaaggga gacagaagca acaagaggaa gaggcgggct
14761 attgagggat cacattccca gaggaaagga ggagctggag agcctgggtg gagggaagac
14821 tcctcctggg aggtagaggg caaagaagcc agctgttaga gacacattta caggtggcag
14881 agaagctgga ggcactccta tctgccacct gatccattcc tccttcactg cccctaagca
14941 ggaatccaac cctagctggt ctcattgccc attccacagc aactgcccag tgcctcacct
15001 ctcagatcaa ccattgaggc aggaatggag acaagatgac cccaagggct tttcttctcc
15061 ctagttcaat ggttttatga tacaaactac tgacatacgt ttttcaagtt attttctcct
15121 tcttctagga aatcccttct gagtgatgtc acatcttggc aggggtggag gagagcctgg
15181 ttgcccaggg atttgtcctt ggggacatct catccatcaa gttgcacact cactggcatc
15241 tttgctatgg ggacattcca atttgcactt tcaggaacac tctgaattcc aagtagaatt

FIG. 18 (cont.)

15301 gatttccctt cttctgtcat ctacctttc tcttcatttt cccattttta ttacccttct
15361 ttccatttct ctctccagtc ttccacctgg aagccctctc tggctaagga caggcaggtg
15421 cccctctctc catcagagga cacctgtact ggagagcaac acaggatggt ctctgccatg
15481 aactggaggc caggaatctc ctcactgaaa attacagtat ggtaactttg caaatggtgg
15541 ttgtttcttc caagactcca gccctgattg cgcaaaactg aaaggcatgt gaagggaagg
15601 aagaggaaga gtgcaaaaca ttgaagagag agctgagtga gctgaagagt gaggatatga
15661 gtagccccaa cccaaacctg gagatgggga gaaacctaca gaatactagc cagagctcct
15721 ccttgtcttg gcagcctact agggacctgg ggaagcaaaa acgaaagctg ggcaacatgc
15781 ctgctttaga atgttttcct tctacttaca catcttccac aggtctcaga atctttcctt
15841 cctctcatcc ttttctccta tctacatatc tatcagagta tccactgttt attcaacaac
15901 tactacttga tggtcagaca caaacaaaca agctaggtgc taattaataa agatacgagt
15961 tttggccggg tgcggtggct cacgcctgta atcccagcac tttgggaggc cgaggcgggc
16021 gaatcacgag gtcaggagtt caagaccagc ctggccaaca tggtgaaacc ccatctctac
16081 taaaaataca aacaattaac tgagcatagt ggtgggcacc tataatacca gctactccgg
16141 aggctgaggc aggagaatcg cttgaaccca ggaggcagag gttgcagtga gctgagatcg
16201 cgccactgca ctctagccgg agtgacagag taagactctg tctcaaaaat aaataaataa
16261 ataaataaat aaataaataa ataaataaaa aataataata caagttttca taagcacact
16321 tctaacccct tgtcttttat gtatttcctt ccttatccac gcacctgtct ccctctactc
16381 cagcctcatt accccagagg tcagtcctca ggaaaactaa acacaaagaa agagctcagt
16441 cagaaaggcc atttatttat gtttcaagat gctcactgcc tcctttgttt tgtctccttt
16501 gcaggccttc tctcttaggc ctcttctcct gggggtatgg atcctggggg gagattgatc
16561 acctccatgc ttccattcct ccccagccat agtggggaca tcatgagaga agccaagcca
16621 ctggcccagg atcacccggc atttatggtg gctgctctgg cacaggtcct tgcctttata
16681 gcccctccag tgatccataa ggccctcttt ctccccaaag gagaggtcac agatagggca
16741 aaggtagctc ttctgcttcc agtgggtctg ctggtgtctg accagcctgg aaaatgagct
16801 gaaagacttg ctgcaatgga agcagtagtt gggcggctct gtgaggtggc ccttctggtg
16861 tctggagaga taggatttct tgctaaaagt caaagaacaa tgggggcaac agaagacatt
16921 gagtcttgag ggcttcactg gatgagagtt ggatctggca tcctgacaga gggttccagt
16981 gatgggtgcc tgggtcctgg tcacaggtgc ttggttctta agtacagatg cctggttctg
17041 ggccatagga ccctcagttc taaatatggg ttcctgggac ctggccactg gtgcatggtt
17101 cacatccaaa agcccctgga tggacctctg gcttctggcg atgggtgtct ggaattcagc
17161 ctgggtgcct ggaatcctca aagtacactc ctggtttcca tccactggct cctggttttg
17221 gtgtatcttc tggtggcgtt tgagctcaga ctggtcccgg aagctcttcc cacacacaga
17281 gcatgaatgg ggccggtaac ccagatggac gcggcggtga cgacttagtc cagaagcatc
17341 acagtaggtc ttgtcacaga gcgtgcaaca gaagggcctc tccccaagat gcatgcgtct
17401 gtgatagctg agggacttgg ggctccgaaa caacttccca cactgactgc agctgttagt
17461 cagcttggga ttgtgaacaa actggtggct atagaggtag gagcgcctgc tgaaacattt
17521 ggcacaggtg tagcaaaa

FIG. 18 (cont.)

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  1 tttgtatgtc attgcaggat tcatgctttc cagtgtgtca tctatggaac tgcctctttc
 61 ttcttccttt atggggccct cctgctggct gagggcttct acaccaccgg cgctgtcagg
121 cagatctttg gcgactacaa gaccaccatc tgcggcaagg gcctgagcgc aacggtaaca
181 gggggccaga aggggagggg ttacagaggc caacatcaag ctcattcttt ggagcgggtg
241 tgtcattgtt tgggaaaatg gctaggacat cccgacaagg tgatcatcct caggattttg
301 tggcaataac aaggggtggg gggacaa
```

FIG. 19

1 ctgtatcagt gctcctcgtc gcctcactgt acttcacgga agagacttgg ttgactggcc
61 acttggagcg gaatcaggag acattcccaa ctcagagaga ctgagcccta gctcgcccac
121 ttgctggaca agatgatatt ccttaccacc ctgcctctgt tttggataat gatttcagct
181 tctcgagggg ggcactgggg tgcctggatg ccctcgtcca tctcagcctt cgagggcacg
241 tgtgtctcca tcccctgccg tttcgacttc ccggatgagc tcagaccggc tgtggtacat
301 ggcgtctggt atttcaacag tccctacccc aagaactacc cgccagtggt cttcaagtcc
361 cgcacacaag tggtccacga gagcttccag ggccgtagcc gcctgttggg agacctgggc
421 ctacgaaact gcaccctgct tctcagcacg ctgagccctg agctgggagg gaaatactat
481 ttccgaggtg acctgggcgg ctacaaccag tacaccttct cggagcacag cgtcctggac
541 atcatcaaca cccccaacat cgtggtgccc ccagaagtgg tggcaggaac ggaagtagag
601 gtcagctgca tggtgccgga caactgccca gagctgcgcc ctgagctgag ctggctgggc
661 cacgaggggc taggggagcc cactgttctg ggtcggctgc gggaggatga aggcacctgg
721 gtgcaggtgt cactgctaca cttcgtgcct actagagagg ccaacggcca ccgtctgggc
781 tgtcaggctg ccttccccaa caccaccttg cagttcgagg gttacgccag tctggacgtc
841 aagtaccccc cggtgattgt ggagatgaat tcctctgtgg aggccattga gggctcccac
901 gtcagcctgc tctgtggggc tgacagcaac ccgccaccgc tgctgacttg gatgcgggat
961 gggatggtgt tgagggaggc agttgctgag agcctgtacc tggatctgga ggaggtgacc
1021 ccagcagagg acggcatcta tgcttgcctg gcagagaatg cctatggcca ggacaaccgc
1081 acggtggagc tgagcgtcat gtatgcacct tggaagccca cagtgaatgg gacggtggtg
1141 gcggtagagg gggagacagt ctccatcctg tgttccacac agagcaaccc ggaccctatt
1201 ctcaccatct tcaaggagaa gcagatcctg gccacggtca tctatgagag tcagctgcag
1261 ctggaactcc ctgcagtgac gcccgagcac gatggggagt actggtgtgt agctgagaac
1321 cagtatggcc agagagccac cgccttcaac ctgtctgtgg agtttgctcc cataatcctt
1381 ctggaatcgc actgtgcagc ggccagagac accgtgcagt gcctgtgtgt ggtaaaatcc
1441 aacccggaac cctccgtggc ctttgagctg ccttcccgca acgtgactgt gaacgagaca
1501 gagagggagt ttgtgtactc agagcgcagc ggcctcctgc tcaccagcat cctcacgctc
1561 cggggtcagg cccaagcccc accccgcgtc atttgtacct ccaggaacct ctacggcacc
1621 cagagcctcg agctgccttt ccagggagca caccgactga tgtgggccaa aatcggccct
1681 gtgggtgctg tggtcgcctt tgccatcctg attgccattg tctgctacat cacccagaca
1741 agaagaaaaa agaacgtcac agagagcccc agcttctcag cgggagacaa ccctcatgtc
1801 ctgtacagcc ccgaattccg aatctctgga gcacctgata agtatgagag tgagaagcgc
1861 ctggggtccg agaggaggct gctgggcctt aggggggaac cccagaact ggacctcagt
1921 tattccccact cagacctggg gaaacgaccc accaaggaca gctacaccct gacagaggag
1981 ctggctgagt acgcagaaat ccgagtcaag tga

FIG. 10

1 masqkrpsqr hgskylatas tmdharhgfl prhrdtgild sigrffggdr gapkrgsgkd
61 shhpartahy gslpqkshgr tqdenpvvhf fknivtprtp ppsqgkgrgl slsrfswgae
121 gqrpgfgygg rasdyksahk gfkgvdaqgt lskifklggr dsrsgspmar r

FIG. 21

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  1 mglleccarc lvgapfaslv atglcffgva lfcgcgheal tgtekliety fsknyqdyey
 61 linvihafqy viygtasfff lygalllaeg fyttgavrqi fgdyktticg kglsatvtgg
121 qkgrgsrgqh qahslervch clgkwlghpd kityaltvvw llvfacsavp vyiyfntwtt
181 cqsiafpskt sasigslcad armygvlpwn afpgkvcgsn llsicktaef qmtfhlfiaa
241 fvgaaatlvs lltfmiaaty nfavlklmgr gtkf
```

FIG. 22

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  1 maslsrpslp sclcsfllll llqvsssyag qfrvigprhp iralvgdeve lpcrispgkn
 61 atgmevgwyr ppfsrvvhly rngkdqdgdq apeyrgrtel lkdaigegkv tlrirnvrfs
121 deggftcffr dhsyqeeaam elkvedpfyw vspgvlvlla vlpvlllqit lglvflclqy
181 rlrgklraei enlhrtfdph flrvpcwkit lfvivpvlgp lvaliicynw lhrrlagqfl
241 eelrnpf
```

FIG. 23